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PHYSICO-CHEMICAL PROPERTIES OF THE ENZYMES INVOLVED IN SHRIMP MELANOGENESIS

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(Manuscript received June 15, 1959)

Practical methods of melanosis prevention *(1, 8, 9, 10)* in shrimp have been extensively studied, but inadequate data have been reported concerning physical and chemical properties of the enzyme(s) involved in the conversion of phenolic derivatives to melanins following death of shrimp. Oxidative enzymes from a large variety of sources catalyze reactions leading to melanin formation *(2, 6, 7, 14, 17, 18, 21)*, and these are generally referred to as tyrosinases or phenol oxidases (phenolases).

Impure preparations of phenol oxidases (phenolases) may be distinguished from other oxidases through their ability to catalyze two essentially different oxidations: the insertion of an hydroxyl group into monophenols *ortho* to the one already present (oxygen transferase activity), and the oxidation of *o*-dihydric phenols to their corresponding *o*-quinones *(20)*. Most impure preparations have both activities in the presence of trace quantities of *o*-dihydric phenols. The question of whether the same or different enzymes catalyze the oxidation of mono- and dihydric phenols has stimulated much research and speculation but remains unsettled.

Where the enzyme has been sufficiently purified, homogeneity studies by electrophoretic and sedimentation methods indicate that only one protein moiety is implicated in both types of oxidation and catalyzed by phenolase prepared from the common edible mushroom *Psalliota campestris (18)*, but no evidence is available to indicate whether or not the activities of enzymes from other sources are due to a single protein or to two or more protein moieties. Due to the lack of data concerning the specificities of these oxidizing systems, they are sometimes referred to as phenolase complexes *(19)*.

The data presented in this paper describe properties of the enzyme(s) involved in shrimp melanogenesis and are in general similar to those for certain phenolases cited in the above references. These properties include stability of the enzyme(s) and the effects of temperature, substrate level and co-factors on activity.

METHODS AND MATERIALS

I. Materials. Crude enzyme extracts used for experiments were press juices from either *Penaeus setiferus* or *Penaeus aztecus* heads. The juices were obtained by pressing contents from heads in a press similar in design to a Carver press. Specific explanation will be given of treatments of these press-juice samples where appropriate.

II. Enzyme activity determinations. A. *Colorimetric methods.* 1. Pyrocatecholase activity. Pyrocatecholase activity of various shrimp preparations was determined colorimetrically by a method similar to that of Smith and Stotz *(22)* for determining phenolase activity of plant material. Hydrogen and palladium were used to reduce 2, 6-dichlorobenzene indophenol and the leuco dye then re-oxidized in the presence of 9×10^{-5} M pyrocatechol and enzyme.

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The procedure used was as follows: 5 ml aliquots of 0.0005 M leuco dye and 0.1 M phosphate buffer (pH 6.0) were transferred to Evelyn tubes, mixed and immersed in a constant temperature bath maintained at 35° ± 0.1 C. This mixture was shaken several times during the next 5 min and 1 ml of enzyme solution added followed by the addition of 1 ml of 0.1 M pyrocatechol (35° C). Contents of the tube were mixed quickly by swirling and the tube was inserted in an Evelyn photoelectric colorimeter (660 mmc filter) and the lamp rheostat adjusted to 100% transmittancy. Simultaneously, a stop watch was started, and the tube immediately returned to the 35° C water bath. The mixture was shaken after 30 sec to replenish oxygen and the galvanic reading recorded after 60 sec. Similar procedures were followed to measure autoxidation of pyrocatechol and endogenous oxidation of the enzyme. For these determinations the enzyme and pyrocatechol respectively were replaced by 1 ml of water.

Relative catecholase activity was computed by converting percentage transmittance after one minute to absorbance, substracting the sum of the autoxidation of substrate and endogenous oxidation of enzyme from total oxidation and multiplying by 10^3.

Absolute or specific activity for pyrocatecholase as measured by this method was based either on quantity of non-dialyzable solids (mg/ml) or on the amount of protein (mg/ml). It was computed by dividing these quantities into the relative activity.

2. Dopa (3,4-Dihydroxyphenyl-alanine) oxidase activity. Dopa oxidase activity was also determined by a colorimetric procedure. The method depended on the oxidation of dopa to a red compound, 2-carboxy-2, 3-dihydroindole-5, 6-quinone (dopachrome) which is relatively stable at pH 6.0.

The procedure was as follows: Seven ml of 0.2 M sodium phosphate buffer (pH 6.0) and 2 ml of 0.5% (w/v) gelatin were added to Evelyn tubes and warmed to 35° C. Two ml of substrate containing 3.5 mg DL-dopa per ml were added followed by 1 ml enzyme solution and the samples maintained at 35° C for 3 hours. At the end of the reaction period, the absorbance was determined photometrically in an Evelyn colorimeter at 470 mmc and compared with a blank containing enzyme, buffer and water. No correction was necessary for autoxidation of the substrate at pH 6.0.

Relative dopa oxidase activity was computed by converting percentage transmittance obtained after three hours to absorbance, subtracting endogenous oxidation of enzyme and multiplying the result by 10^3.

Specific activity for dopa oxidase was obtained by dividing the relative activity either by non-dialyzable solids (mg/ml) or amount of protein (mg/ml).

B. Manometric method. *1. Phenolase activity.* Oxygen uptake was measured in standard Warburg manometers using flat bottom 20 ml reaction flasks. One ml of solution containing the required amount of substrate, 1.9 ml of sodium phosphate buffer (pH 6.8) and 0.5 ml of 1% gelatin solution were added to the reaction flask. Potassium hydroxide was placed in the center well. Enzyme solution (0.5 ml) was pipetted into a side arm, the manometers and flasks connected to the water bath and allowed to oscillate 70 oscillations per minute for 5 min. The system maintained at 25° C was then closed, the enzyme mixed with substrate and manometer readings were taken at zero time. Subsequent readings were taken at 5 min intervals for 20 min, then at 10 min intervals for 1 hr or more depending on the activity of the enzyme.

Oxygen uptake curves were linear during the early stages of oxidation and the slopes of these curves were used as measures of enzyme activity. Exogenous oxidation of the added substrate was determined by substracting the sum of the endogenous oxidation of the enzyme and autoxidation of substrate from total oxidation. Relative activity was based on exogenous substrate oxidation. Specific activity was based on mcl oxygen uptake per hr per mg of non-dialyzable dried (95° C) solids.

III. Methods for determining effect of heating on shrimp. *1. Pyrocatecholase activity.* Effect of heating for 1 min at temperatures between 40° C and 80° C was determined on 2 shrimp-head samples. One sample was prepared by pressing juice from the heart and stomach area of *P. aztecus;* the other was a leucocyte-free sample from the pericardial sinus area (including heart) of *P. setiferus.* Juice of *P. aztecus* was frozen, thawed, and centrifuged (2,600 × *g*, 20 min) and the supernatant decanted. The top layer of the sediment contained numerous blood corpuscles and this was resuspended in the supernatant. The sample from *P. setiferus* was prepared by grinding hearts with sand in 0.1 M sodium phosphate buffer (pH 6.0), freezing, thawing, centrifuging and dialyzing. The dialysate was diluted 1 to 5 with cold copper-free water, filtered through

a 0.5 cm thick pad of celite (L-665 filter aid) and finally centrifuged ($4,500 \times g$, 10 min). There were no leucocytes in the supernatant.

Three-ml aliquots of these samples were added to Evelyn tubes containing 15 ml of 0.2 M sodium phosphate buffer (pH 6.0) and heated to 40, 50, 60, 70, 75, and 80° C as follows: tubes were immersed in a water bath and the solutions gently stirred with a thermometer and the appropriate temperature was maintained for 1 min. The tubes were then immersed in an ice bath and stirred to hasten temperature lowering. Samples were then examined individually at 35° C for pyrocatecholase activity by the indophenol method.

The thermal stability of shrimp pyrocatecholase was determined by heating at 35° C for periods of time from 30 min to 7 hr. An aqueous extract of *P. aztecus* heads was lyophilized and 3 g of the lyophilized powder ground to a paste with 5 ml of distilled water and dispersed in 25 ml of distilled water. This was centrifuged ($2,600 \times g$, 10 min) and a portion of the supernatant incubated at 35° C with 0.2 M sodium phosphate buffer (pH 6.0) in the proportion of one part enzyme to 5 parts buffer (maintained at 35°C prior to mixing). Two 6-ml aliquots were removed after 30 min and after 1 hr and then hourly for 7 hr. Each sample was immediately cooled in ice water following its removal from the water bath and kept cool until analyzed for pyrocatecholase activity by the indophenol method.

IV. Methods for determining energies of activation. *1. Manometric method.* The energy of activation was determined manometrically for shrimp pyrocatecholase by the method of Gould *(13)* with modifications as outlined under manometric methods. The pyrocatechol concentration in the reaction mixture was 2.0×10^{-3} M.

Press juice from the gill area of shrimp heads was frozen and thawed several times and then centrifuged ($27,000 \times g$, 10 min) to remove lipids, pigments and insoluble protein. Five 20 ml portions were frozen and later thawed as needed to determine enzyme activities at temperatures ranging from 10° C to 30° C.

2. Colorimetric method. The indophenol method was used also to determine the energy of activation of shrimp pyrocatecholase. The sample for this determination was prepared and stored by the procedure outlined in the above section. Activities were determined at 10° C, 20° C and 30° C and an Arrhenius plot made to determine the energy of activation.

V. Method for determining Michaelis constants. Enzyme preparations used for determining Michaelis constants were partially purified from *P. aztecus* heads as follows: the heads were blended in an equal weight of cold distilled water in a Waring blender and the resulting solution strained through 5 layers of cheese cloth. This extract was then frozen, thawed and centrifuged ($5,400 \times g$, 20 min) and the supernatant filtered through a ¼ inch pad of celite. The filtrate was lyophilized and the dried material ground to a fine powder. A 3% dispersion (w/v) was centrifuged ($2,600 \times g$, 10 min) and the supernatant used to determine Michaelis constants.

The indophenol and manometric methods were used to determine pyrocatecholase activities at substrate levels of 1×10^{-1} M to 5×10^{-3} M and the colorimetric method used to determine dopa oxidase activities at substrate levels between 1×10^{-2} and 1.25×10^{-3} M.

VI. Procedure for determining effect of copper on pyrocatecholase activity. It was found by an experiment similar to that of Kubowitz *(15)* that the removal of copper brought about a decrease in shrimp pyrocatecholase activity and that its addition reactivated the enzyme.

Press juice from *P. setiferus* heads was centrifuged ($27,000 \times g$, 10 min), dialyzed for 24 hr against copper-free water and a small portion frozen. The remainder of the solution was dialyzed against 0.1 M sodium cyanide followed by 24 hr dialysis against copper-free water. The dialysate was divided into 3 equal portions and frozen for several days. All 4 samples were thawed, and each of the 3 cyanide treated samples was supplemented with one of the following solutions: 0.5 ml of copper sulfate containing 250 mcg Cu^{++}; 0.5 ml containing 10 mcg Cu^{++}; and 0.5 ml of copper-free water. Pyrocatecholase activity was then determined manometrically in the presence of 2×10^{-3} M pyrocatechol.

RESULTS

Effect of temperature on the pyrocatecholase activities of the 2 enzyme preparations is shown in Figure 1, where positions 1 and 2 indicate the relative activities of the

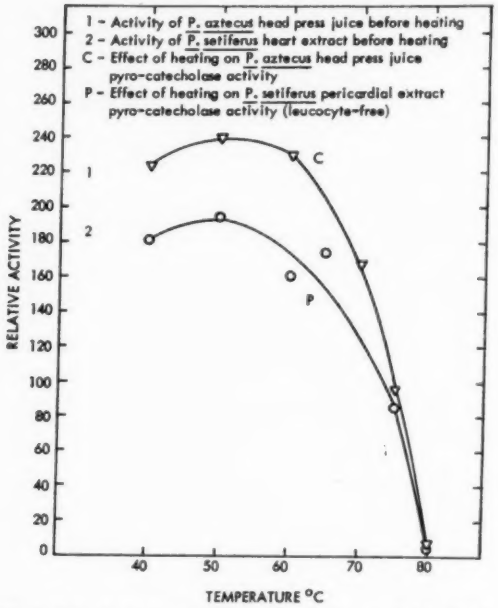

Figure 1. Effect of heat on pyrocatecholase activity of shrimp-head press juice and heart extract.

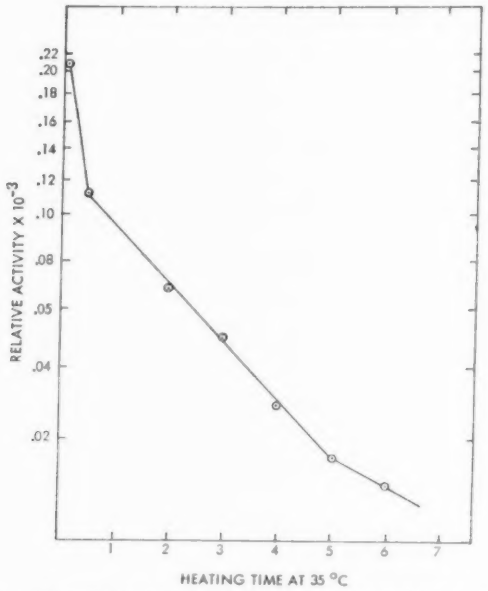

Figure 2. Thermal inactivation of shrimp pyrocatecholase at 35° C.

preparations prior to heating (i.e. 211 and 182, respectively). It can be noted from these curves that heating the crude preparation for 1 min between 40° C and 60° C resulted in increased pyrocatecholase activity. Maximum increase was obtained at a temperature of 50° C and amounted to 12.3% for the leucocyte-containing sample and 4.4% for the leucocyte-free sample. When the latter was held for 2 min at this temperature the increase in activity was 26%.

The straight line obtained by plotting the logarithm of relative pyrocatecholase activity against heating time at 35° C is shown in Figure 2. The inactivation of pyrocatecholase activity after heating at this temperature between 30 min and 6 hr was approximately first order, since the relationship of time vs activity decreased linearly during this period. The enzyme was inactivated almost completely after 7 hr under these conditions and its half-life was approximately 1 hr.

Figure 3 is an Arrhenius plot showing the effect of temperature on pyrocatecholase activity obtained by manometric and colorimetric methods. From these curves the values of energy of activation were calculated by using the Arrhenius equation: $-E = 4.58 \times$ slope, and were 3,000 calories/mole and 5,200 calories/mole, respectively.

The following table presents the results of the effect of copper ions on oxygen uptake in the presence of *P. setiferus* press juice and pyrocatechol.

Treatment	Oxygen uptake mcl/min
Non-treated	5.2
Cn^- treated + water	0.9
Cn^- treated + 250 mcg copper	3.7
Cn^- treated + 10 mcg copper	3.2

Michaelis constants were determined graphically from double reciprocal plots [1/v versus 1/(S)] according to the method of Lineweaver and Burk *(16)* (Figure 4). A value of 3.65×10^{-3} moles/l. was found by the indophenol method for pyrocatecholase activity and 5.0×10^{-3} moles/l. by the manometric method. The constant for dopa oxidase activity was 3.78×10^{-3} moles/l.

DISCUSSION

The effect of temperature on pyrocatecholase activity shows that the enzyme can be activated when subjected to certain temperatures for short periods of time. Since an active phenol oxidase is believed to be present in shrimp blood cells, it is possible that enzyme released from these cells caused increased activity after heating.

This does not explain, however, the increase in pyrocatecholase activity of the sample which contained no leucocytes. In view of the extensive investigations of Bodine and co-workers *(3–5)*, it is possible pyrocatecholase in shrimp samples is proenzymatic in nature and active centers are exposed by heating. Results from preliminary experiments concerning the effects of different inorganic salts on shrimp pyrocatecholase activity also supports the existence of phenolase as a zymogen in shrimp extracts. The presence of pyrocatecholase as a proenzyme in shrimp would partly explain the increased incidence of melanosis in shrimp kept at air temperatures for long periods prior to refrigeration *(11)*.

The activation energy is valuable for interpreting reaction mechanisms. If a crude extract such as those employed in these studies attacks several chemically related substrates, the question might arise whether the extract contains only one or several enzymes. If the activation energies of the extract on all substrates is constant, then only one enzyme is present. Values obtained for the activation energies of shrimp pyrocatecholase suggest a high activity for this enzyme and also support the belief that this phenolase is an

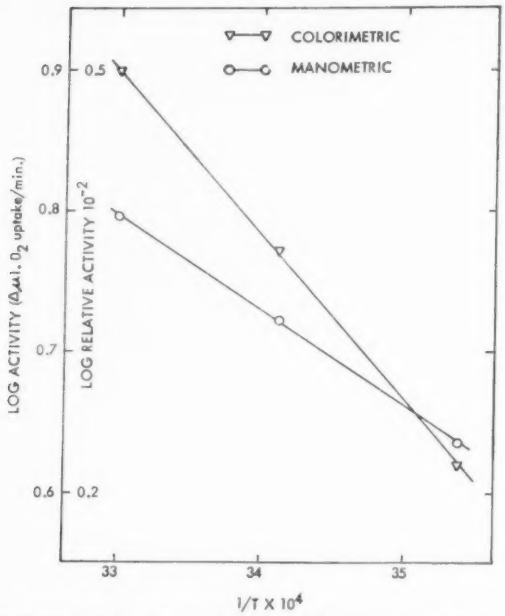

Figure 3. Arrhenius plots of catecholase activities measured by colorimetric and manometric methods.

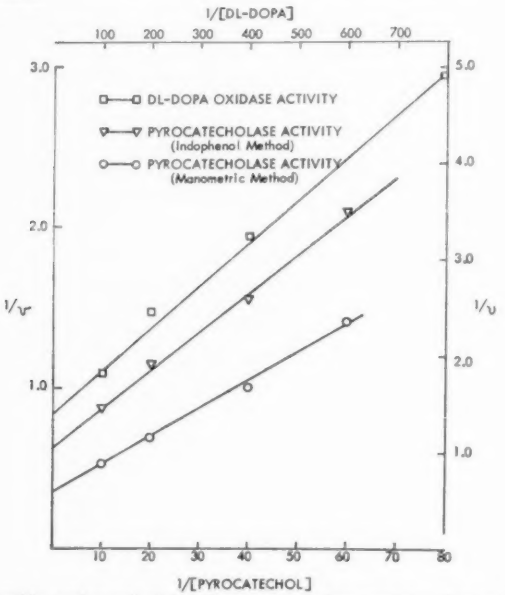

Figure 4. Double reciprocal plots of pyrocatecholase activity determined by colorimetric and manometric methods.

oxygen transferase (aerobic dehydrogenase) rather than an anaerobic dehydrogenase *(20)*. This is evidenced by the fact that most anaerobic dehydrogenases have activation energies ranging from 15,000 to 24,000 cal/mole *(12)*. Gould *(13)* reported a value of 2,700 cal/mole as the activation energy of phenolase preparations from Irish potato, mushroom *(Psalliota campestris)* and meal worm *(Tenebrio molitor)*, acting either on pyrocatechol or *p*-cresol. This suggests a close similarity of phenolase from various sources and that a single enzyme is involved in both the mono- and *o*-dihydric phenolase activities of these enzymes.

The Michaelis constant is a measure of the substrate concentration required to give half the maximum velocity of an enzyme under definite conditions and is considered by many investigators as a constant for enzymes from different sources that catalyze the same reaction. Any factor that varies the velocity constant for combination of enzyme and substrate such as pH, ionic strength, temperature, etc., will also vary the Michaelis constant. Then, substrate level at half-maximum activity can be used to characterize an enzyme provided conditions are rigidly controlled and specified. The constants reported here can be used in future studies to characterize the shrimp enzyme(s), but no valid comparison can be made between these constants and those previously reported in the literature or any constants derived under conditions different from those outlined above. The two constants for pyrocatecholase activity are in good agreement considering the differences in the two methods used to determine activity.

Another important factor in the activity of shrimp pyrocatecholase is its requirement for copper. All enzymes thus far identified as phenolases by various investigators required copper for their activity. This co-factor is specific *(14)* and activity is lost when copper ions are replaced by other metal ions.

Results reported here demonstrate the necessity of copper for activity of shrimp pyrocatecholase, and this was further substantiated in this laboratory (unpublished results) where various copper-sequestering agents as diethyldithiocarbamate, thiourea and cysteine were shown to inhibit shrimp pyrocatecholase activity.

SUMMARY

This paper reports some of the physico-chemical characteristics of the enzyme(s) involved in shrimp melanogenesis. Evidence is presented which indicate that shrimp pyrocatecholase is a zymogen which can be activated by heating under controlled conditions. The inactivation rate of this enzyme at 35° C is first order and its half-life is approximately one hour under these conditions.

Activation energy values identify shrimp pyrocatecholase with a group of phenolases referred to as oxygen transferases (aerobic dehydrogenases) rather than anaerobic dehydrogenases and also reveal that this enzyme is very active in reference to the activities of certain other enzymes.

Michaelis constants were determined for shrimp pyrocatecholase by both the manometric and colorimetric methods and for dopa oxidase by a colorimetric method, but since the conditions of these experiments were different from those used to determine these constants for similar enzymes, no direct

comparisons can be made. These constants may be useful for future characterization of similar enzyme systems.

It was shown that copper ions are essential for the activity of shrimp pyrocatecholase and this is also characteristic of phenolases as a group.

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PHENOL OXIDASE IN SHRIMP AND CRAB

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The major difficulty encountered in the purification of phenolases is the relatively small quantities of active material present in comparison to total inactive components. The best source of phenolases appears to be mushroom *(Psalliotes campestris)* where only 5 to 10 mg of enzyme per lb of wet material has been found *(11)*, and enzyme from this source has considerably greater activity than shrimp enzyme(s).

In an effort to locate the area in shrimp and the specie of shrimp containing the highest concentration of phenolase, a study was made of enzyme activities of various anatomical areas of different species of shrimp including antennae, abdominal shell, press juice from various areas of the cephalothorax and blood. Thorough studies were made of phenolase activities in blood samples from *Penaeus aztecus*, *Penaeus setiferus*, and the blue crab *(Callinectes sapidus)*. Experiments were also carried out to determine natural substrates involved in shrimp melanosis and effects of certain oxidative inhibitors on the catecholase activity of shrimp protein.

MATERIALS AND METHODS

A. Shrimp samples analyzed. The *P. setiferus* exoskeletons, taken from freshly caught shrimp, were frozen until analyzed for phenolase activity. The *Penaeus duorarum* exoskeletons were taken from shrimp that had been refrigerated in ice approximately one week prior to analysis. The exoskeletons were taken from the abdomen of shrimp and included the thin pellicle which separates the epidermis from the remainder of the shell.

Juice was pressed from fresh *P. aztecus* and *P. setiferus* heads, centrifuged (4,500 x *g*, 10 min) and frozen until analyzed. Blood samples were obtained by aspirating blood from the pericardial sinuses of live shrimp or immediately following their death. If freshly caught shrimp are chilled to approximately 15° C immediately after death, their blood remains uncoagulated for 1 hr or more. Blood samples were collected from shrimp of different catches by inserting a No 20 hypodermic needle through the thin membrane which separates the carapace from the abdomen, into the pericardial area and aspirating the blood into small polyethylene bottles.

Some of these blood samples were treated with various antioxidants and anticoagulants and centrifuged to separate the corpuscles from the plasma. Other samples were centrifuged after the blood had coagulated. The various samples were then fractionated as described in the results and analyzed for phenolase activity *(3)*.

B. Preparation of crab blood samples. Large crabs *(C. sapidus)* were kept at 4° C for 1 hr and blood removed by severing one or two crawling legs between the first and second joints. The colorless blood was collected in a cold polyethylene beaker and stirred to remove fibrinogen. This blood turned light blue upon exposure to air as a result of oxidation of the hemocyanin. It was then centrifuged (27,000 x *g*, 20 min) to remove residual clot and cells.

A 50-ml aliquot of the serum was used to further purify hemocyanin. It was chilled to 0° C and cold 5% acetic slowly added until a slight precipitate appeared at pH 4.8. This was then dialyzed against distilled water at 0° C for 72 hr, and water removed from the dialysate by freezing. The solute in this type of solution lowers the freezing point and water is frozen on the surface of the container. As the temperature of the

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solution is lowered the protein is concentrated in the center of the cake. After freezing and thawing a blue viscous, water-insoluble precipitate settled to the bottom of the flask. This was washed several times with copper-free water and dispersed in 0.1 M sodium phosphate buffer (pH 6.0). The clot from the original blood was suspended in buffer and frozen. It was then ground with sand in a mortar, frozen and thawed several times and finally centrifuged.

Another hemocyanin fraction was prepared by adjusting an aliquot of the serum to pH 4.8, dialyzing, then freezing and thawing. After removing most of the residual water in vacuum, a blue crystalline material remained which contained numerous anisotropic, micaceous crystals of various sizes. These crystals were insoluble in water and only slightly soluble in dilute salt solutions. A small portion (1 mg/ml) could be dissolved in 0.1 M sodium phosphate buffer (pH 6.0).

C. Determination of phenolase activity. Colorimetric and manometric methods were used to determine the various phenolase activities. The manometric procedure used for determining phenolase activities was similar to that described by Bailey, Fieger and Novak *(2)*. Duplicate 0.25 g samples of shell or antennae and 0.5 ml samples of press juice or blood were added to Warburg flasks and buffered with 0.5 M sodium phosphate buffer (pH 6.8). Potassium hydroxide was used to absorb carbon dioxide. The substrates (in 1 ml of copper-free water) were added from side arms after shaking in an open system at 25° C for 5 min. Activities were computed from the straight portion of curves obtained by plotting oxygen uptake against time.

Endogenous oxidation in the absence of substrate was subtracted from total oxidation (oxidation in the presence of added substrate) and the reaction rate computed. Specific activity was based on the quantity of oxygen (microliters) taken up per g of dried material per hr. Dry weights were determined by drying 10-g samples of the various material at 95° C for 12 hr.

The colorimetric methods used for determining pyrocatecholase and dopa oxidase were those described by Bailey *et al (2)*.

D. Protein determination. Either the biuret method of Hiller, McIntosh and Van Slyke *(8)* for determining protein in urine or the sulfosalicylic acid method of Exton *(6)* was used when the quantity of enzyme sample was limited. Ovalbumin, isolated by the method of La Rosa *(9)*, was used as a standard for both of these methods and the protein values determined by the sulfosalicylic acid method converted to equivalent quantities of biuret protein so that a more direct comparison could be made in determining specific activities. One mg of protein determined by the sulfosalicylic acid method was equivalent to 1.67 mg of protein determined by the biuret method when egg ovalbumin was used as a standard. Specific activities were obtained by dividing relative activities by the mg of protein per ml of sample.

E. Methods for determining natural substrates. In an effort to identify natural substrates involved in melanin formation of shrimp, a paper chromatographic procedure similar to that of Bailey *(1)* was used to separate the phenolic compounds in samples of press juice from heads and from blood of *P. setiferus*. Ninhydrin *(5)*, modified Folin and Ciocalteu phenol reagent *(10)* and fluorescence after treatment with ammonia were used to locate phenols on the paper chromatograms. The organic fraction of a mixture of 1-butanol, acetic acid and water (40:10:50) was used as solvent in an ascending paper chromatography chamber.

A 20-mcl aliquot of the various samples along with standard phenolic compounds was applied to Whatman No 1 filter paper. The chromatograms were placed in solvent-saturated chambers and the solvent allowed to ascend the paper for 12 hr. The air-dried chromatograms were dipped in the appropriate developing reagent, dried at room temperature and heated at 85° C for 5 min.

F. Methods used to study inhibition of shrimp. *Pyrocatecholase and dopa oxidase.* Chemical inhibition of both pyrocatecholase activity and endogenous oxidation of shrimp head press juice was determined by manometric and indophenol methods. The method used to determine inhibition depended on the character of the inhibitor. Inhibition of dopa oxidase activity by some compounds was determined by the colorimetric procedure described by Bailey *et al. (2)*.

The material obtained by pressing the juice from *P. aztecus* heads was frozen (-40° C) and thawed several times and the supernatant used for the various studies. The inhibitors were incorporated in the buffer and the degree of inhibition determined by comparing the activities of these samples with inhibitor-free samples.

RESULTS

A. Phenolase activity of shrimp shell and antennae. Data concerning substrate concentration, source of enzyme and oxygen uptake for shrimp phenolase preparations in the presence of various substrates are presented in Table I.

Enzyme in abdomen shell and its adhering pellicle from *P. setiferus* and *P. duorarum* catalyzed the oxidation of both mono-and *o*-dihydric phenols, but catalyzed oxygen uptake to a greater extent in the presence of the dihydric phenols. Enzyme from *P. duorarum* shell catalyzes the oxidation of dihydric phenols (DL-dopa and pyrocatechol) more rapidly than that from *P. setiferus* shells, but the cresols are apparently oxidized at a slower rate by protein from the former source. Hydroquinone was oxidized at a very rapid rate by *P. duorarum* shells.

Antennae from *P. aztecus* exhibited both DL-dopa oxidase and *p*-cresolase activities. This material apparently supplied an excellent source for phenolase(s) but due to its association with macromolecules, could not be fractionated.

B. Pyrocatecholase activity of shrimp head press juice and extracts. The press juice from *P. aztecus* catalyzed the oxidation of both mono- and *o*-dihydric phenols but catalyzed the oxidation of DL-tyrosine at a greater rate than it did an equal-molar quantity of DL-dopa, indicating a relatively high monophenolase activity. As shown in Table 1, press juice from *P. aztecus* heads catalyzed the oxidation of L-dopa at approximately twice the rate of equal-molar quantities of DL-dopa, and catalyzed the oxidation of *p*-cresol at an exceedingly high rate.

Numerous technics were employed in attempts to concentrate and purify enzyme from press juice *(3)*, but the maximum increase in purity achieved through use of these methods was 17-fold, represented by a pyrocatecholase specific activity of 130. Since this value, as pointed out later, is lower than pyrocatecholase activities obtained from certain shrimp blood fractions, blood is obviously a better initial source for fractionation studies.

C. Phenolase activity of shrimp blood. *P. setiferus* blood catalyzed the oxidation of both mono- and *o*-dihydric phenols, and the oxidation of L-dopa approximately twice as fast as equal-molar quantities of DL-dopa.

Blood appeared to be a good source of phenolase(s) and this was substantiated by further experiments. When fluid was pressed from freshly caught *P. aztecus* heads and

TABLE 1

Oxygen uptake for enzyme preparations in the presence of various substrates

Source of enzyme	Shrimp specie	Substrate and concentration	Oxygen uptake mcl./g./hr
Abdomen shells	*P. setiferus*	DL-dopa (C_3)	270
		pyrocatechol (C_5)	260
		p-cresol (C_6)	100
		DL-tyrosine (C_4)	50
	P. duorarum	DL-dopa (C_3)	350
		pyrocatechol (C_5)	304
		p-cresol (C_6)	68
		m-cresol (C_6)	54
		hydroquinone (C_2)	408
Antennae	*P. aztecus*	DL-dopa (C_3)	480
		p-cresol (C_6)	504
Press juice	*P. aztecus*	L-dopa (C_1)	220
		DL-dopa (C_1)	100
		pyrocatechol (C_1)	260
		DL-tyrosine (C_1)	180
		p-cresol (C_1)	720
		hydroquinone (C_1)	300
Blood	*P. setiferus*	L-dopa (C_1)	330
		DL-dopa (C_1)	165
		p-cresol (C_1)	330
		hydroquinone (C_1)	125

$C_1 = 2.0 \times 10^{-3}$M; $C_2 = 3.4 \times 10^{-3}$M; $C_3 = 5.0 \times 10^{-3}$M; $C_4 = 5.5 \times 10^{-3}$M; $C_5 = 9.1 \times 10^{-3}$M; $C_6 = 9.3 \times 10^{-3}$M.

frozen and thawed, a large quantity of clotted blood was observed in the mixture. When the clots were removed, homogenized by grinding with sand, centrifuged and dialyzed, the pyrocatecholase activity of the dialysate was 58. This was more active than any other nonfractionated preparation examined by the colorimetric method for pyrocatecholase activity.

In a further experiment, hearts were removed from approximately 200 freshly caught *P. setiferus*, dispersed in 10 ml of 0.1 M sodium phosphate buffer (pH 6.0), frozen and thawed, ground with silica, centrifuged and the supernatant dialyzed. The dialysate had a pyrocatecholase activity of 80 and a DL-dopa oxidase activity of 92.

Extracts from sediment obtained by centrifuging clotted blood samples catalyzed the oxidation of pyrocatechol and DL-dopa at greater rates than any other samples from shrimp examined by the colorimetric methods for phenolase activities. The most active preparation for pyrocatecholase was found in the sediment obtained by centrifuging clotted blood aspirated from *P. aztecus*. This sediment was dispersed in water, frozen and thawed several times and sonorated to lyse blood corpuscles. This extract was filtered through celite to produce a filtrate whose pyrocatecholase activity was 155. The DL-dopa oxidase activity of this sample was 70. The original centrifugate was treated similar to the sediment to produce an extract whose phenolase activities for pyrocatechol and DL-dopa were 36 and 13, respectively.

A relatively active dopa oxidase was obtained from *P. setiferus* blood by a fractionation method similar to that described above. The sediment obtained after centrifuging was frozen and thawed, ground with sand, filtered through celite and then dialyzed. The resulting dialysate contained enzyme(s) whose activity as pyrocatecholase and dopa oxidase were respectively 92 and 121. The centrifugate from the original sample was filtered through celite and dialyzed to produce a dialysate whose phenolase activities for pyrocatechol and DL-dopa were 58 and 13.

Both heparin and oxalic acid apparently prevented coagulation of shrimp blood, but oxalic acid irreversibly inhibited phenolase activity since the activities of blood fractions after treatment with this material were appreciably lower than non-treated samples. Dialyzed fractions of blood from *P. aztecus* treated with a mixture of heparin and sodium bisulfite contained phenolase(s) whose activities were approximately equal to nontreated samples. The sodium bisulfite retarded blackening which occurs during centrifugation of non-treated samples.

D. L-tyrosine, a natural substrate in shrimp melanogenesis. L-tyrosine was found in all samples analyzed by the chromatographic procedure. These samples included shrimp blood, press juice from heads and dilute acetic acid extracts of shells. L-dopa was also present in several blood samples analyzed. No other phenols were identified in these samples by the methods used.

Pressed juice from *P. aztecus* heads was diluted 1 to 10 with 0.4 M sodium phosphate buffer and one mg/ml of DL-dopa was added. Oxygen was bubbled through this solution for 2 min and the flasks stoppered. The solution turned red-brown after 2 hr at 25° C and absorbed light strongly at 305 mmc and 475 mmc. After 5 hr at this temperature the solution turned black. This same sequence of color changes was descibed by Mason *(12)* for the 3 chromophoric phases brought about by the action of meal worm tyrosinase on dopa. The material absorbing strongly at 305 mmc and 475 mmc was characterized by him as hallochrome red (dopa-chrome). When diluted shrimp head press juice was kept under similar conditions in the absence of added dopa, the same sequence of color changes occurred and the red-brown solution formed absorbed strongly at 475 mmc. Absorbance at this wave length indicated the presence of hallochrome red.

E. Pyrocatecholase activity of crab serum and hemocyanin. The original crab serum fraction along with the extract from the sediment (cells and clot) and the dilute solution of hemocyanin crystals were examined for pyrocatecholase activities. The serum had a pyrocatecholase activity of 18 while the processed sediment and the solution of the hemocyanin crystals both had pyrocatecholase activities of 22.

Manometric determination of pyrocatecholase activity revealed that crab serum catalyzed oxygen uptake in the present of 3.7×10^{-1} M pyrocatechol to the extent of 208 mcl/min at pH 6.8 and 25° C, but did not catalyze the oxidation of saturated hydroquinone, *p*-phenylenediamine, *p*-cresol, L-tyrosine or L-dopa. The dilute sodium phosphate extract of the cystalline crab hemocyanin had no mono-phenolase activity. It did not catalyze the oxidation of L-dopa, *p*-cresol, or L-tyrosine.

F. Inhibition of pyrocatecholase and dopa oxidase in shrimp head press juice. Dopa oxidase activity of *P. aztecus* head press juice was inhibited 95% in the presence of

TABLE 2

Chemical inhibition of catecholase activity and endogenous oxidation of shrimp head press juice

Compound	pH[1]	Method	Percent inhibition	
			(Total)[5]	(Endogenous)[6]
Substances which combine with copper				
Diethyldithio-carbamate (0.017 M)	6.0	Manometric[4]	36	70
Thiourea (0.02 M)	5.9	Manometric[4]	35	30
Cysteine (0.012 M)	6.0	Manometric[4]	30	33
Carboxy acids or their salts				
Sodium EDTA[2] (0.0002 M)	6.5	Indophenol	45	50
Sodium benzoate (0.002 M)	6.6	Indophenol	46	23
Calcium acetate (0.012 M)	6.0	Indophenol	7	
Oxalic acid (0.002 M)	5.6	Manometric	97	27
Substance that complexes phenols				
TAT-21[3] (0.002 M)	6.0	Manometric	46	1

[1] pH after reaction.
[2] (Ethylenedinitrilo) tetra acetic acid-(sodium salt).
[3] Triethanolamine titanate.
[4] Manometric determinations made as outlined in the presence of 4×10^{-2}M pyrocatechol.
[5] Inhibition of total oxidation in presence of added pyrocatechol.
[6] Inhibition of endogenous oxidation (no pyrocatechol added).

0.01 M ascorbic acid. It was inhibited 86% in the presence of 0.005 M sodium bisulfite and was completely inhibited in the presence of 0.01 M cysteine.

Results showing the degree of inhibition of various other chemicals on total oxidative activity in the presence of pyrocatechol and on endogenous oxidation of shrimp head press juice are given in Table 2.

DISCUSSION

The preferential oxidation of *o*-dihydric phenols in the presence of shrimp abdomen shells and other shrimp phenolase preparations indicate that L-dopa, as is the case with chordates *(13)*, is the most probable natural substance of this enzyme complex. In view of the discussions of Mallette *(11)* and other investigators, the oxidation of monophenols is catalyzed by certain phenolase preparations only in the presence of dihydric phenols which can be derived from mono-phenols by autoxidation. The absence of dihydric phenols in some shrimp preparations and the presence of these substances in other preparations might account for the differences in the rates of oxidation of *p*-cresol and other mono phenols. Naturally occurring *o*-dihydric phenols would enhance the oxidation of mono phenols by certain preparations such as from shrimp antennae.

The increased dopa oxidase activity of *P. duorarum* shells compared to that of *P. setiferus* shells accounts for the poor keeping quality of the former species of shrimp in regard to melanogenesis. *P. duorarum* are noted for rapidly developing "black spots" soon after being caught even when kept at refrigerated temperatures, although rapid handling and refrigeration following catching lengthens the storage life of these shrimp.

Antennae from *P. aztecus* would provide an excellent source of shrimp phenolase if it were more easily removed from associated substances. Several technics useful in dissociating other enzymes from inactive components including ultrasonic vibrations, organic solvent fractionation and freezing and thawing were used in unsuccessful attempts to purify phenolase(s) from antennae. This enzyme complex is possibly involved in the quinone hardening of these tissues by reactions similar to those outlined by Mason *(13)* for the Arthropod cuticle. The process of sclerotization would prevent the separation of active phenolase from other cuticular components.

Manometric results of oxidations of dopa in the presence of shrimp head extract and blood indicated that shrimp phenolase may be specific for the L-isomer of this compound. The concentrations of dopa used in these experiments were considerably lower than those required for maximal activity and activity is approximately doubled for each 2-fold increase in substrate concentration until the substrate level reaches 2.5×10^{-2} M, *(2)*. Since the DL mixture contained approximately equal quantities of the two isomers, the results indicate that D-dopa was only slightly oxidized. Samples of D-dopa were not available for more exact studies in regard to the optical specificity of shrimp dopa oxidase.

Pressed juice from shrimp heads contained not only blood but also cellular fluids from internal organs as liver and stomach. The most active preparations were obtained by pressing juice from the gill area of shrimp heads. These juices, consisting principally of blood, appear to be the principal source of enzyme(s) involved in melanogenesis.

Sufficient quantities of blood for thorough fractionating studies were difficult to obtain, but since blood contains considerably greater quantities of extractable phenolase than any other shrimp preparation, this source will undoubtedly be the most important one for further studies of this enzyme.

During the examination of numerous blood samples it was found that the sediment obtained after centrifugation contained the most active phenolases. These results point to the presence of phenolase(s) in shrimp blood corpuscles and was further substantiated by certain properties of shrimp protein exhibited during fractionation. Freezing and thawing, as well as lyophilization tended to increase the total pyrocatecholase activity of extracts (predominately blood) from shrimp heads. High-speed centrifuging of pressed juices left a gel-like residue layered over the bulk of the insoluble protein which had higher specific activity than the remainder of the protein in the sediment or the supernatant. Storing at 0° C followed by thawing also increased total activity of some preparations.

A comprehensive histological study of crustacean blood corpuscles was made by Hardy *(7)* who found two distinct types of corpuscles in crab blood. Both kinds were amoeboid and both resembled the white rather than the red corpuscles of mammalian blood. One of these types was a stable, highly refractive granular cell and appeared to be identical to eosinophile granules. The other type consisted of extremely sensitive cells which exploded upon contact with a foreign body such as glass.

Two different types of blood corpuscles are also present in shrimp blood. Both types are clearly visible after staining with Wright's or Giemsa stains. One of these types is mononucleated and resembles small mammalian lymph-

ocytes. The other type consists of granulated mononucleated cells which are slightly larger and which contain more cytoplasm than the lymphocyte type. The larger cells are the most unstable of the two and great numbers of these are apparently ruptured when blood smears are made. Ruptured cells were observed in both stained and unstained blood specimens. It is possible that these cells are similar to the "Explosive cell" of Hardy and they may contain protein which catalyzes the oxidation of phenols. This postulation is supported by the work of Pinhey *(14)* who found explosive cells in the blood of *Cancer pagurus* which appeared to be associated with phenolase activity.

The presence of L-tyrosine and L-dopa in shrimp blood samples would supply natural substrates for the production of melanins by the presence of phenolase complex. The apparent formation of hallochrome red indicates that the three chromophoric phases of Mason occur during the formation of melanins in shrimp enzyme preparations all support the idea that the black materials which form in shrimp after death are dopa melanins.

Results from studies of crab *(C. sapidus)* blood indicates that phenolase is absent in the corpuscles of this species, but that hemocyanin is an active pyrocatecholase. The phenolase activity of this material was specific since it did not catalyze the oxidation of other phenolic compounds. Similar results were reported by Bhagvat and Richter *(4)* who also found that purified hemocyanin from crab *(Cancer pagurus)* catalyzed the oxidation of pyrocatechecol.

These findings are further evidence for the existence of two different enzyme systems in crustacean blood which catalyze the oxidation of phenols. The most active enzyme is present in the corpuscles and catalyzes the oxidation of both mono- and *o*-dihydric phenols while hemocyanin from blood is specific for the oxidation of pyrocatechol. The most important enzyme contributing to shrimp melanogenesis appears to be enzyme released from blood leucocytes.

The *in vitro* inhibition of dopa oxidase by ascorbic acid and sodium bisulfite supplement data from previous experiments which indicated the usefulness of these materials in the practical control of shrimp melanosis.

Sodium bisulfite is presently being used commercially for delaying black spot development in ice-stored shrimp. Only the high cost of cysteine prevents its use in the commercial prevention of melanosis. This material acts as a reducing agent and as a copper-sequestering agent and both are important in preventing this discoloration. Ascorbic acid is presently too expensive for commercial use by the shrimp industry.

Reducing substances such as the sulfites, ascorbic acid and cysteine are useful in the control of black spots in shrimp because they immediately reduce *o*-quinone and retard melanin formation. Sulfhydryl and other reducing groups and reducing enzymes present in shrimp may serve to maintain the redox potential sufficiently low in live shrimp to prevent melanosis. This possibly explains why exoskeletons do not blacken in live shrimp although substrate and enzyme are present.

SUMMARY AND CONCLUSIONS

Some of the characteristics of phenol oxidizing enzyme(s) found in shrimp *(P. setiferus, P. aztecus* and *P. duorarum)* and the blue crab *(C. sapidus)* are described. Attempts were made to locate the specific areas in shrimp as well as the specie of shrimp containing the highest concentration

of phenol oxidase (phenolase). Phenolase(s) were partially purified from shrimp head press juice and blood and these were used to determine substrate specificity and the natural substrate for the enzyme(s) involved in shrimp melanogenesis as well as the effect of various classical phenolase inhibitors on these materials.

Shrimp phenolase catalyzes the oxidation of both mono- and *o*-dihydric phenols and is present in the exoskeleton and adhering epicuticle, in the antennae and in the blood. Orthodihydric phenols are oxidized more readily than monohydric phenols in the presence of most shrimp preparations examined.

The most active shrimp phenolase(s) are found in blood and enzyme(s) from this source catalyze the oxidation of both mono- and *o*-dihydric phenols. Protein which catalyzes the oxidation of monophenols and dopa is apparently localized in blood leucocytes and is stereospecific for the L-isomer of dopa. Crystalline hemocyanin from *C. sapidus* catalyzes the oxidation of pyrocatechol but not other phenols and this may be true for shrimp hemocyanin.

Free L-tyrosine was found in all shrimp samples analyzed and appears to be a natural substrate necessary for melanin formation in shrimp. The oxidation of L-tyrosine and L-dopa to dopa-chrome and other chromophores in the presence of shrimp extracts identify the melanin formed as dopa melanin.

Several groups of chemical compounds including substances which combine with copper, carboxy acids, reducing agents and phenol-sequestering agents inhibit dopa and pyrocatechol oxidation. Usefulness of ascorbic acid and the sulfites in retarding shrimp melanogenesis was substantiated.

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CHANGES IN HYDRATION AND CHARGES OF MUSCLE PROTEINS, DURING FREEZE-DEHYDRATION OF MEAT[a]

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Freeze-drying is the mildest method known for drying meats *(5, 15, 18, 20)* but even this process causes undesirable changes of meat quality. The texture of lyophilized and rehydrated meat is drier than the frozen control *(17)*. The decrease of tenderness and juiciness produced by freeze-drying apparently is caused by a loss of the water-holding capacity of muscle proteins *(3, 7)*. Hamdy *et al (7)* pointed out that the dry texture and the decrease of meat hydration is one of the principal problems in the field of lyophilization of meat.

In a previous paper *(4)* it was shown that quick-freezing of muscle tissue does not decrease the hydration of muscle nor cause protein denaturation. Therefore, the influence of freeze-drying on the water-holding capacity of meat is not due to the freezing process but to the process of dehydration.

The studies reported here concern the kind of biochemical changes in meat proteins caused by freeze-dehydration. It may be that dehydration causes a certain denaturation of muscle proteins since it has been shown that some proteins are denatured by lyophilization *(14)*. The possibility of denaturation is indicated by the fact that the adenosinetriphosphatase activity of actomyosin is partially reduced by freeze dehydration of beef muscle *(13)*. It is possible to study the denaturation of meat by means of the determination of the water-holding capacity and of the buffer capacity at different pH values and the measurement of the amount of dyes bound by the acidic and basic groups of muscle proteins *(11)*. These methods have been adapted to study the influence of freeze-drying on meat proteins.

This report concerns only the effect of freeze dehydration and not the storage of dried meat. Only the changes in muscle tissue free of extraneous connective and fatty tissue were studied. Therefore, the conditions of these experiments do not correspond exactly to the commercial application of freeze-drying of meat.

METHODS

The animals used were U. S. Utility 5- and 6-year old cows. Two to 3 lb of the *longissimus dorsi* muscle were cut out from the carcass 5 to 6 days after slaughter. Connective tissue and fat were removed to the extent possible. The meat was ground or cut into cubes in the manner previously described *(4)*.

Freeze-drying. Two-hundred grams of meat, usually in cubes, were put in the 4 vessels of a laboratory free-drying apparatus. These were placed in an acetone solid carbon dioxide mixture for 15 min and then attached to the apparatus. Gooding and

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Rolfe *(5)* reported that meat which has been rapidly frozen failed to give products which reconstitute easily. On the other hand, Wang *et al (18)* found that rehydration does not depend on the pre-freezing temperature. For the present work, freezing at −55° C was selected because at higher freezing temperatures (e.g., −15° C) the freezing process itself causes a decrease of hydration *(4)*. Drying required 24–30 hr and at the end of this time the pressure was 40–50 mc Hg, and the temperature 20–25° C.

Rehydration. The amount of water necessary to reach the original amount of moisture was calculated as follows:

$$m_d = \frac{(100 - w_d)}{100} \cdot m \quad \text{and} \quad W_a = \frac{m_d \cdot w_f}{100 - w_f}$$

where m = weight of dried meat in grams; w_d = moisture content of dried meat, %; m_d = dry matter in m; w_f = moisture in fresh meat and w_a = added water for rehydration in milliliters. The dried meat was mixed thoroughly with added water by using a spatula and kept in a refrigerator (2° C) for 30 min.

When the meat was dried in the ground state, no further treatment was used. When the meat was dried in cubes, the rehydrated meat was ground in the same manner as the fresh meat. The water that was not adsorbed during rehydration was added to the ground meat after the first grinding. In this technique of rehydration the relative position of fibers, which is important for the rate of rehydration of meat pieces *(1)*, is not important.

Analysis of fresh and rehydrated meat. All methods have been described: determination of moisture *(11)*; water-holding capacity, determined by pressing of muscle on filter paper at room temperature *(6)*; water-holding capacity after heating at 70° C (tube method) *(4, 19)*; influence of pH on the water-holding capacity of meat containing 60% added water (press method), and determination of the isoelectric point *(9)*; buffer capacity of muscle tissue and of the water- and trichloracetic acid (TCA) -extracts of meat at different pH values *(10)*; determination of basic and acidic groups in muscle by binding of dyes *(11)*; determination of proteins in meat *(11)*; and determination of pH values in muscle and in the juice of the "tube method" *(11)*.

RESULTS AND DISCUSSION

Influence of freeze-drying on the water-holding capacity of tissue at the natural pH value of meat. According to the results of the press method, the freeze-drying causes in all meats a marked decrease in water-holding capacity (Table 1). Most of the samples of dried meat appeared to take up the added water completely or nearly completely, yet the absorbed water in rehydrated meat is not bound as strongly as in the fresh tissue. Therefore, the adsorption is not a good criterion for the quality of freeze-dried meats.

When the water-holding capacity of fresh and rehydrated meat was determined by the tube method (heating at 70° C), essentially no differences were noted. Obviously, the effect of heating is equal to or more severe than the effect of freeze-drying. Occasionally, freeze-drying causes a shift of the meat pH but this shift is not significant.

Table 2 shows water-holding capacity of fresh and rehydrated meat with different amounts of added water. For both of these experiments the meat was freeze-dried in ground state; the dried material was powdered in a mortar; and water was added for rehydration. In spite of the great surface area of the powdered dried meat, its hydration is lower than the hydration of the fresh muscle.

Influence of the temperature of rehydration on the water-holding capacity of meat. Auerbach *et al (1)* did not find any influence of the temperature of rehydration-water on the rate and level of rehydration in the range between 22 and 55° C. Hamdy *et al (7)* used a temperature of 40–

TABLE 1

Influence of freeze-drying on the water-holding capacity and the pH of muscle tissue at the normal (natural) pH of meat

Meat sample No	pH of fresh meat	Moisture of fresh meat	pH of rehydrated meat	Moisture of dried meat	Moisture of rehydrated meat	State of meat before rehydration	Water-holding capacity of meat			
							Press method (60% added water)		Tube method (heat at 70° C)	
							Fresh	Rehydr.	Fresh	Rehydr.
I	5.56	74.34	5.58	4.92	74.70	powdered	58.3	47.0	.....	.....
II	5.57	69.60	5.58	4.02	69.81	powdered	48.2	30.0	.....	.....
III	5.46	74.13	5.48	6.48	74.03	powdered	44.3	28.9	38.2	29.2
IV	5.57	72.60	5.62	2.41	72.61	cubes	56.2	41.2	38.2	34.2
V	5.50	72.81	5.49	3.40	72.80	cubes	50.9	40.7	41.2	37.2
VI	5.52	73.20	5.63	6.06	72.75	cubes	41.7	31.0	39.2	.....
VII	5.38	73.21	5.49	3.85	71.30	cubes	36.4	26.7	35.4	36.5
VIII	5.70	74.45	5.52	4.54	74.12	cubes	41.7	29.4	.....	.....
IX	5.62	74.59	5.48	3.87	74.61	cubes	47.6	34.8	38.5	38.6

TABLE 2

The water-holding capacity (press method) of fresh and rehydrated meat at different amounts of added water

Meat No	Added water %	Fresh meat Bound water in % of meat	Rehydrated meat Bound water in % of meat
II	0	51.4	48.0
II	20	52.8	49.6
II	40	47.2	43.4
II	60	48.2	30.0
III	0	49.4	30.0
III	20	50.9	36.0
III	40	49.6	36.0
III	60	44.3	28.9

50° C for rehydration. In those experiments commercially dehydrated pieces of meat containing fat were used. Therefore, it was necessary to check the influences of the temperature of rehydration on lean meat.

Meat cut in cubes was freeze-dried and rehydrated (a) with water at 27° C, temperature after rehydration, 25° C; (b) with water at 50° C, temperature after rehydration, 36° C. Both rehydrated meat samples had the same content of moisture. Table 3 shows a remarkable drop of muscle

TABLE 3

Influence of temperature of rehydration on the water-holding capacity of meat by the press method

Added water %	Bound water in % of meat		
	Fresh meat	Rehydrated 27° C	Rehydrated 36° C
0	55.0 (38.2)[1]	49.0 (34.2)[1]	42.0 (34.2)[1]
20	56.0	50.4	43.6
40	55.7	50.0	38.3
60	56.2	41.2	32.6

[1] In parentheses are the results by the tube method at 70° C.

hydration with an increase of temperature. That is not surprising because denaturation of meat proteins begins at temperatures >30° C, resulting in a decrease of hydration at the normal pH of meat *(11)*. For this reason, in all following experiments the rehydration was carried out at room temperature.

The tube method indicated no influence of the temperature of rehydration. The heating of meat involves such strong changes of water-holding capacity *(11)* that an effect of the temperature of rehydration is no longer observed.

Influence of the shape of meat on the water-holding capacity of rehydrated meats. It was necessary to determine the best method of drying which excludes all factors influencing meat hydration other than those of freeze-drying. An important factor is the shape of meat.

Meat was dried after cutting into cubes and after grinding. The dried ground meat was broken into small pieces and powdered in a mortar. After rehydration, the cubed meat was ground in the same manner as the fresh meat. With regard to the extent of drying and the moisture of dehydrated meat,

there were no noticeable differences among the various samples. The water-holding capacity of all samples was less than that of fresh meat, but there are characteristic differences in the samples. The hydration of the powdered ground meat was greater than that of the rehydrated and ground cubed meat, and this in turn was somewhat higher than hydration of the ground dehydrated meat which was rehydrated without powdering. (Table 4). Thus the grinding of meat before drying is disadvantageous apparently because the meat dried in ground state is more compact than the meat dried in cubes.

TABLE 4

Influence of the shape of meat on the water-holding capacity of rehydrated meat. (press method)

Added water %	Bound water in % of meat			
	Fresh meat	Rehydrated sample A[1]	Rehydrated sample B[2]	Rehydrated sample C[3]
0	50.7 (41.2)[4]	44.3 (37.2)[4]	44.3 (36.2)[4]	47.7 (37.3)[4]
20	54.9	41.2	40.0	46.9
40	51.0	44.4	40.2	48.6
60	50.9	40.7	39.8	42.2

[1] A = Ground, dried, then broken in pieces.
[2] B = Dried in cubes, ground after rehydration.
[3] C = Ground, dried, then powdered.
[4] In parentheses are results of tube method, 70° C.

By powdering, the surface area of dried meat is enlarged; therefore, its hydration is higher than that of unpowdered material. The differences of hydration between powdered and unpowdered samples show the importance of the surface area for the hydration of meats. By powdering the dehydrated meat the meat is affected by conditions which are not comparable with those found when fresh meat is ground, dehydrated and then rehydrated.

For the study of the influence of freeze-drying on meat proteins all additional effects, such as those caused by different conditions of grinding, must be prevented. The preferred method may be drying in cubes and grinding after rehydration.

The tube method (heating at 70° C) indicates differences corresponding to the results of the press method but the magnitude of the differences is much less.

Influence of pH on the water-holding capacity of fresh and rehydrated meat. Determining the effect of pH on the water-holding capacity of meat, measured by the press method, makes it possible to determine the isoelectric point (I.P.) of muscle and certain relative changes of protein charges. The interpretation of the pH-hydration-curves with regard to the I.P. and to the negative and positive charges of the muscle proteins were discussed in a previous paper *(9)*.

Results obtained by investigation of 4 different meats yield similar characteristic differences between fresh and rehydrated meat. Figure 1 shows the data for one meat. No marked shift of I. P. (minimum of hydration always about pH 5.0) is observed, as in the case of heated versus fresh meat *(11)*. Decrease of meat hydration (as determined by the press method) caused by drying occurs only in the pH range from 4.5 to 6.5. The maximum decrease of the water-holding capacity is at approximately

pH 5.0, the I.P. of muscle. At pH 6.0–6.5 and pH 4.5 the rehydrated meats have the same ability of hydration as the fresh muscle. At pH >6.5 the hydration of rehydrated meats is greater than that of fresh meats.

These results indicate that in the isoelectric range of dried meat less charged protein groups are available for binding water than in the fresh muscle. Apparently, freeze-drying results in a more closed structure of proteins. But this tighter network cannot be caused by an irreversible formation of new stable cross linkages between the peptide chains as disulfide-, peptide- or ester bonds because by addition of acid or base the hydration of tissue is restored. Probably during freeze-drying a change of the orientation of polypeptide chains occurs in such a manner that these chains lie more closely together after drying than before. The close structure may be stabilized by new salt cross linkages between carboxyl and amino groups or/and new hydrogen bonds, causing a steric hindrance for water adsorption.

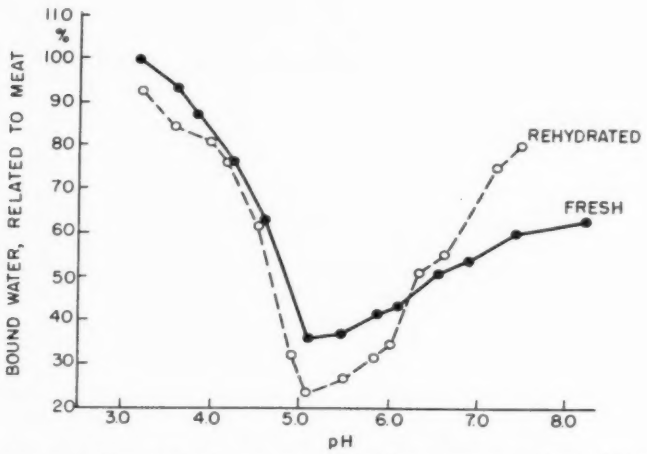

Figure 1. Influence of pH on the water-holding capacity of fresh (●) and rehydrated (○) meat.

The amount of available acidic groups in the rehydrated meat is greater than in the fresh muscle because the increase of hydration in the basic range of the I.P. caused by freeze-drying increases with increasing pH (Fig. 1). But there is no increase of basic groups in the acidic range of I.P.

Influence of pH on the buffer power of fresh and rehydrated meat. The change of the buffer power dA/dpH or dB/dpH (A=meq. added acid, B=meq. added base) post mortem and the participation of the single components of muscle (structural proteins, water soluble proteins and non-proteins) on the buffer power of the total tissue are discussed in a previous paper *(10)*. In these experiments the buffer power is related to 100 g of meat.

The buffer power of fresh and rehydrated meat was calculated from the titration curves of tissue. The analysis of 4 different meats indicates similar characteristic differences between fresh and rehydrated meat (Table 5). In

the pH range from 6 to 7 the buffer capacity of the rehydrated meat is higher than that of fresh meat. In the pH range 5.0 to 3.5 the differences between both fresh and rehydrated meat are relatively small. At pH 3.5 the differences were greater but no clear result was observed. A better view of the effect of drying is given by the buffer capacity of the structural proteins.

TABLE 5

The influence of pH on the buffer power of different fractions of fresh and rehydrated meat

Buffer power, $\frac{dA}{dpH}$ or $\frac{dB}{dpH}$, related to 100 g muscle

pH	Meat M	Water extract W	TCA extract T	Total protein M-T	Water soluble protein W-T	Structural proteins M-W
Fresh meat						
6.31	4.92	3.54	3.20	1.72	0.34	1.38
6.80	5.46	3.99	3.60	1.86	0.39	1.47
7.10	6.43	3.94	3.62	2.81	0.32	2.49
7.42	6.39	3.82	3.48	2.91	0.34	2.57
8.30	5.00	3.14	2.87	2.13	0.27	1.86
5.12	6.21	3.00	.....	.....	.....	3.21
4.65	9.52	3.70	.....	.....	.....	5.82
4.22	10.82	4.98	.....	.....	.....	5.84
3.81	11.63	6.40	.....	.....	.....	5.23
3.59	12.31	6.70	.....	.....	.....	5.61
3.12	12.40	7.32	.....	.....	.....	5.08
Rehydrated meat						
6.43	6.59	3.44	3.20	3.15	0.24	3.15
7.05	7.59	3.88	3.62	3.97	0.26	3.71
7.49	6.40	3.72	3.44	2.96	0.28	2.68
7.61	6.22	3.67	3.37	2.85	0.30	2.55
4.98	6.67	3.45	.....	.....	.....	3.72
4.53	10.10	4.52	.....	.....	.....	5.58
4.20	12.12	5.98	.....	.....	.....	6.14
3.50	12.88	7.45	.....	.....	.....	5.43
3.12	13.35	7.90	.....	.....	.....	5.45

For this purpose, we investigated the buffer strength of the total tissue, of the water extract (Figures 2 and 3) and of the TCA-extract (Figure 4). The buffer power of the water extracts of fresh and rehydrated meat is similar. The slightly higher buffer strength of the water extract of rehydrated meat in the pH range 3.0–4.5 and 8.0–10.0 may be due to the higher protein content. The coincidence of the buffer curves of the TCA-extracts in the range of pH 6-9 indicates that, by drying of meat, buffering compounds of phosphoric acid are not released by freeze-drying. Certain differences of buffer power between the TCA-extract of rehydrated muscle and that of fresh tissue in the pH range 9.0–10.5 are related to the free amino acids and peptides in muscle *(10)*.

From the data in Table 5 it may be calculated that at pH 7 in rehydrated meat 52% of the total buffer power is due to proteins (48% in fresh muscle), and 94% of the protein buffer power is due to the structural proteins (89% in fresh muscle). The differences in the buffer power of the whole tissue between fresh and rehydrated meat are caused by changes in the buffer

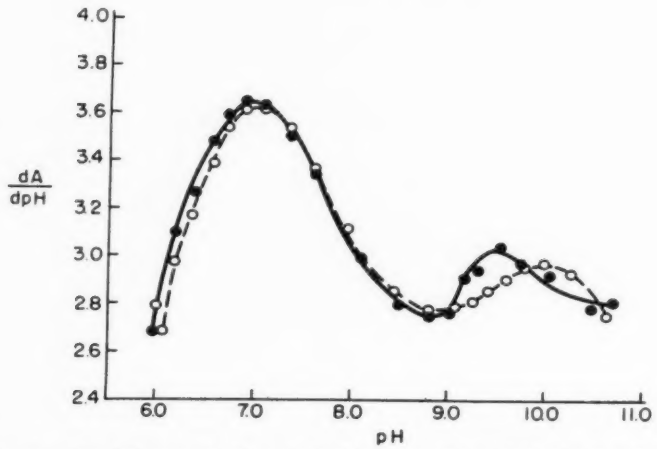

Figure 2. The influence of pH on the buffer power of the trichloracetic acid extract of fresh (●) and rehydrated (○) meat.

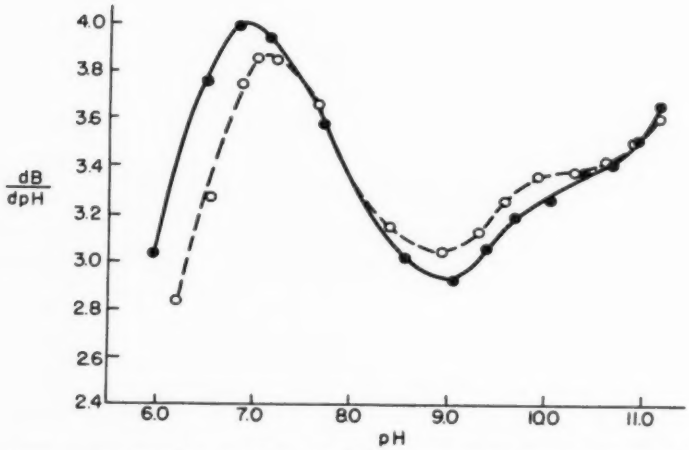

Figure 3. The influence of pH on the buffer power of the water extracts of fresh (●) and rehydrated (○) meat. Basic range.

power of structural proteins during freeze-drying. Figure 5 shows the buffer power, dA/pH or dB/pH, of the structural proteins. There are only small differences in the pH range 3–5, but in the pH range 6–7 the structural proteins of rehydrated meat are stronger buffers than those of the fresh muscle. This increase of buffer power by drying may be caused by an increase of available ammonium groups (α and η) of proteins because the pK values of these groups lie in the range 6–8.

A comparison of these findings with the influence of pH on hydration of meat shows that the amount of available basic groups is decreased only in the basic range of I.P. because freeze-drying causes no increase of hydration in the acidic range of the I.P.

According to the results of the hydration experiments (Fig. 1) it would be expected that the buffer power in the acidic range would increase by drying because of the increase of acidic groups. However, no significant change of buffer power in the acidic range of I.P. occurs during freeze-drying. Therefore, freeze-dehydration seems to cause an increase of acidic groups only in the basic range of I.P.

The determination of protein in the water extracts of tissue indicates that freeze-drying does not result in any decrease of the solubility of globular muscle proteins (Table 6). Also, the amount of water-soluble non-protein compounds is not changed by freeze-drying.

TABLE 6

Influence of freeze-drying on the water solubility of nitrogenous substances of meat

Meat	g non-protein-N in 100 ml extract	g protein-N in 100 ml extract
Fresh	0.253	0.443
Rehydr.	0.252	0.453

Influence of freeze-drying on the shift of pH by cooking of meat. It is known that the pH of meat is increased by cooking *(11)*. This shift of pH (ΔpH) is less in cooking of rehydrated meat than in cooking of fresh muscle (Table 7). According to previous experiments *(11)*, the increase of pH by heating at the normal pH is caused by a decrease of the acidic groups of proteins. On the other hand, the amount of available acidic groups at pH values >I.P. is increased by freeze-drying. Therefore, the drop of

TABLE 7

Influence of freeze-drying on the pH shift effected by cooking of meat

Meat	Fresh meat			Rehydrated meat		
	pH of meat before heating	ΔpH in meat after heating (70° C)	ΔpH in juice after heating	pH of meat before heating	ΔpH in meat after heating (70° C)	ΔpH in juice after heating
IV	5.57	+0.30	+0.33	5.62	+0.18	+0.26
V	5.50	+0.40	+0.40	5.49	+0.26	+0.33
VII	5.38	+0.33	+0.40	5.49	+0.21	+0.23
IX	5.62	+0.34	+0.31	5.48	+0.14	+0.21

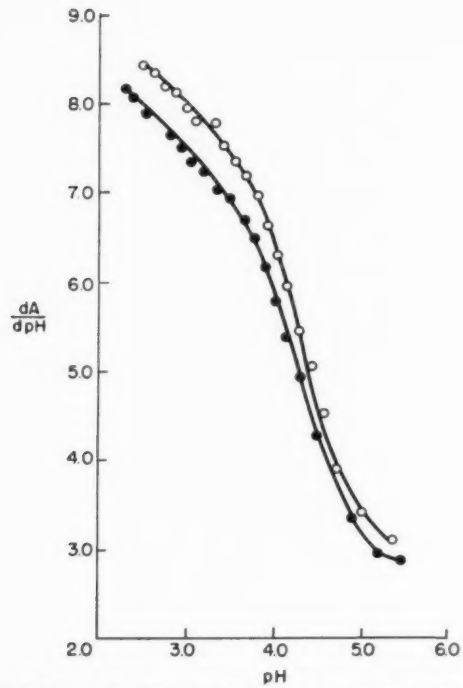

Figure 4. The influence of pH on the buffer power of the water extracts of fresh (●) and rehydrated (○) meat. Acid range.

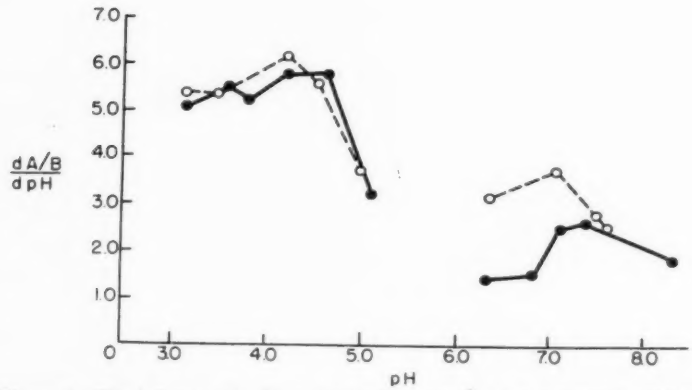

Figure 5. The influence of pH on the buffer power of the structural proteins of fresh (●) and rehydrated (○) meat.

negatively charged groups by heating and consequently the increase of pH will be smaller in the rehydrated muscle than in the fresh tissue.

Influence of freeze-drying on the amount of acidic and basic groups in the muscle proteins. Acidic groups were determined by binding of safranine at pH 11.5, and basic groups were determined by binding of orange G at pH 2.2, as described in a previous paper *(11)*. Table 8 shows that freeze-drying causes a significant increase of acidic groups but no significant change of basic groups. This result agrees very well with that of the determination of water-holding capacity. This is not self-evident because the hydration of muscle was measured in the pH range 3.0 and 7.5 but the dye binding was determined at pH 2.2 and 11.5.

TABLE 8

Influence of freeze-drying on the number of acidic and basic groups of the muscle proteins

Treatment	Equivalents acidic groups per 10^4 g protein	Equivalents basic groups per 10^4 g protein
Fresh	14.7 (0.6)[1]	13.0 (0.2)[1]
Rehydrated	16.0 (0.6)[1]	13.2 (0.3)[1]

[1] Standard deviations shown in parentheses.

It is interesting that a similar, but less marked effect, was observed in fish. Freeze drying of fish muscle results in a similar increase of acidic groups *(3)*. Connel doubted the reliability of his observation but our more marked result and its correspondence with the hydration experiments may confirm his result.

According to other investigators, freeze-drying causes almost no changes of muscle proteins because the total volume of tissue, the appearance of the muscle fibers, the space system between the fibers *(18)*, and the contractibility of the muscle fiber *(12, 13)* are not changed. But techniques used in these investigations show that freeze-drying results in characteristic changes of hydration and charges of muscle proteins.

The decrease of hydration only at pH values in the isoelectric range of muscle is quite similar to the effect of heating at temperatures between 30 and 40° C *(11)*. Therefore, it seems that the tightening of the network of protein structure in this range of pH is caused by an unfolding of peptide chains and a formation of new salt or/and hydrogen bonds which are similar to the first step of heat denaturation. This effect is comparable to the fact that some proteins, particularly myosin, coagulate without previous denaturation. Such proteins are typically very unsymmetrical and, if by any dehydrating process the molecules are brought together, there is sufficient surface common to the two asymmetric molecules to cohere tightly to each other, and a condition of coagulation results *(2)*.

Hydration experiments reported here do not suggest any irreversible formation of new stable cross linkages as mentioned earlier. Therefore, the formation of disulfide bridges between the peptide chains by freeze-drying as it was considered for fish muscle by Connel *(3)* may not occur during freeze-drying of beef muscle.

Apart from the changes in the isoelectric range of pH, there are marked differences between the influence of freeze-drying and the influence of heat denaturation *(11)*. Heating meat up to 40° C causes an unfolding of peptide chains but no change in protein charges. At higher temperatures, the amount of carboxyl groups decreases. No change in basic groups was observed. Freeze-drying, however, results in an increase of acidic groups. This increase of charged groups concerns only the basic range of I.P. In the acidic range of I.P. almost no change of available charged groups occurs during freeze-dehydration. Heating of meat at 40° C results in a decrease of the solubility of globular (and structural) proteins. Freeze-drying does not cause a similar effect. These results indicate a fundamental difference between the effect of freeze-drying and heat denaturation.

The experiments reported here show that freeze-dehydration causes an increase of acidic and probably also of basic groups only in the basic range of I.P. This fact does not support the theory that changes in hydration of meat by freeze-drying are due to the increase of the concentration of ions in the meat juice during dehydration because influences of cations or anions on meat hydration concern both acidic and basic range of I.P. In previous experiments, however, it was found that an exchange or elimination of bivalent cations, which are in meat by nature, increases the hydration only in the basic range of I.P. *(8)*. Therefore, it could be possible that the disorientation of peptide chains during freeze-drying causes an irreversible cleavage of metallic cross linkages (Ca, Mg, Zn). At pH values $<$I.P. this cleavage will have no influence on meat hydration because at low pH values the muscle proteins have a low ability for binding cations *(8)*. But the cleavage of metallic bridges will increase the hydration as the pH increases *(8)*. It was observed that the concentration of total calcium in the water extracts of tissue increases upon freeze-drying *(7)*. Perhaps this calcium, linking the peptide chains of the native muscle tissue, is split off by freeze-drying. Work on this problem is being continued.

According to these results, freeze-drying may cause biochemical changes of muscle tissue by removing water molecules which are localized between the peptide chains and attached to the polar groups of proteins in several layers and bringing together neighboring peptide chains. This approach causes the formation of new salt and/or hydrogen bridges and, therefore, a certain stabilization of a more closely knit structure and a decrease of hydration in the isoelectric range of I.P. Possibly this process is accompanied by an unfolding of peptide chains. This reaction, however, is not identical with the changes occurring during heat denaturation of muscle. The process, which results in a tighter network of protein structure in the range of I.P., also causes a cleavage of cross linkages of bivalent cations and, therefore, an increase of available charged groups and consequently of hydration in the basic range of the I.P.

All these changes apparently concern the submicroscopic structure of muscle because the changes of the histological structure of muscle by freeze-drying are very small *(3, 18)*. On the other hand, the special steric position of the muscle proteins *in situ* within the fiber seems to be important for the changes which we observed during freeze-drying. There is probably only a very little "denaturation" of the single proteins. The extracted pure muscle

proteins, actomyosin and myosin, withstand freeze-drying without marked deterioration of either enzymatic or physiological properties *(16)*.

CONCLUSIONS

Freeze-drying of beef results in a drop of water-holding capacity only in the isoelectric pH-range of muscle. It causes a tighter network of protein structure, probably because the peptide chains after drying lie more closely together. The tighter structure may be stabilized by the formation of new salt and/or hydrogen bonds.

Freeze-drying causes an increase of acidic groups of proteins in the basic but not in the acidic range of the I.P. Probably this increase is due to a cleavage of metallic cross linkages between the protein chains.

The undesirable changes in meat during freeze-drying are not due to the freezing process itself *(4)*. Furthermore these changes are not identical with the alteration of meat by heat denaturation.

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CHANGES IN HYDRATION, SOLUBILITY AND CHARGES OF MUSCLE PROTEINS DURING HEATING OF MEAT[a]

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Heating of meat causes certain physical chemical changes in muscle proteins which affect the quality of cooked meat and meat products. Changes in protein solubility, in the adenosinetriphosphatase activity of myosin, or in the contractibility of the muscle fiber have been used by others to determine the extent of denaturation of meat proteins. In this study the changes in the hydration of muscle were studied because this factor is more directly correlated with the quality of meat *(28, 31, 66, 67)*. It is known that heating releases juice, that the amount of juice depends on the temperature, and that this loss of water influences juiciness and texture of meat *(4, 28, 36, 43, 66, 68)*. In this study we have investigated in some detail the influence of different temperatures on the hydration of beef muscle.

An investigation of the influence of temperature on the pH-dependence of water-holding capacity will give information concerning changes in meat quality and also the mechanism of denaturation. This is because changes in protein net charge and in steric conditions affect meat hydration in a pH range from pH 3.0 to 7.5 *(34)*.

In addition we have determined the acidic and basic groups in muscle and the solubility of the globular and structural muscle protein after heating at different temperature.

METHODS

Utility cows from 4 to 6 years of age were used. Two to 3 lb of the *longissimus dorsi* muscle were cut from the carcass 5–6 days after slaughter. Connective tissue and fat were removed as far as possible. The muscle was ground twice in a cooled meat grinder.

Influence of pH on the water-holding capacity of meat after heating at different temperatures. One hundred g of ground meat at 20° C was minced in a homogenizer with 30 ml ice water at high speed for 30 sec. During mincing the metal homogenizer vessel was cooled by ice.

For heating meat, the covered metal vessel of a homogenizer, filled with 110 g of ground meat, was placed in a water bath of the desired temperature. The meat was heated with occasional stirring until it was at the temperature of the water bath and was kept there for 30 min. [Other authors have shown that after 30 min heating at 70–90° C protein hydration changed very little *(43, 60)*]. Then the vessel with meat was put in ice for about 15 min. Thirty-three ml ice water was added to bring the added water to 30%. The mixture was minced in an ice-cooled homogenizer in the same way as the fresh meat. It was considered that the different texture of heated muscle has an influence on the extent of mincing and, therefore, on the water-holding capacity; however, we found that this effect has no influence on the relative difference of hydration produced by heating.

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In all samples the temperature after mincing did not exceed 20° C. For the determination of water-holding capacity by the "press method," 6.5 g of the homogenate was mixed by spatula with 1.5 ml of distilled water, containing the amount of HCl or NaOH necessary to bring the meat to the desired pH *(34)*.

Influence of the pH of meat before heating on the water-holding capacity after heating at 80° C. The ground meat was minced in a cooled blender for 30 sec. Of this meat 10 g was mixed with 6.0 ml water by spatula. The water contained the amount of NaOH or HCl necessary to bring the meat to the desired pH. After equilibration at 2° C for 30 min, pH and water-holding capacity were determined. The sample was then put into a test tube (2 x 15 cm), closed by a stopper with glass capillary and placed in a water bath at 80° C for 30 min. Next the heated sample was put in ice for about 15 min and after thorough mixing of the tissue with the released juice by spatula, the pH and the water-holding capacity were measured.

Relation of pH and water-holding capacity of raw meat to the amount of juice released on heating. Twenty g of the ground meat was minced with 12 ml of water in an ice-cooled homogenizer for 5 sec. The added water contained the amount of NaOH or HCl necessary to bring the mixture to the desired pH. After measurement of temperature and pH, the water-holding capacity was determined.

Another 20 g of the ground meat was minced with 12 ml water containing NaOH or HCl as described for "raw meat." The mixture was transferred quantitatively into a beaker and weighed. The covered beaker was put in a water bath of 40° C or 80° C for 30 min. The released juice was poured out through a fritted glass disk with large pores. Any pieces of tissue on the disk were reunited with the cooked meat in the beaker. After the beaker plus meat were weighed, 0.50 to 2.00 ml of the released juice was pipetted into a weighed glass dish and dried in an oven at 105° C.

Water-holding capacity of meat heated at different temperatures. Fifty g of ground meat at 20° C was minced with 15 ml water in a small cooled homogenizer for 30 sec. After measurement of temperature and pH, the water-holding capacity was determined.

Fifty g of ground meat was placed in a large test tube with thermometer. Tube with contents was put in a water bath of the desired temperature and kept there for 30 min after the meat reached the temperature of the bath. The tube was then put in ice for about 15 min.

The heated meat, including the released fluid, was transferred quantitatively into a small homogenizer and was minced with 15 ml of ice water for 30 sec. After measurement of temperature and pH, the water-holding capacity was determined.

Determination of water-holding capacity (press method). Water-holding capacity was determined by pressing meat on filter paper according to the method of Grau and Hamm *(23)*. Water equivalent to 60% of the sample was added because the differences of hydration are accentuated when this amount of water is added. Hydration of meat is expressed as per cent of bound water, related to the fresh muscle (without additive). The formula for the calculation of bound water can be applied to cooked meat only up to a limit of 60% added water *(23)*.

Determination of the buffer capacity of muscele tissue, of water extracts and of trichloracetic acid-(TCA-) extracts. Previously reported methods *(35)* were used to determine the buffer power of tissue and tissue-extracts by means of the electrometric titration. Proportion of tissue to extractant (water or TCA) was 1:1. Buffer values for residues and extracts were calculated with respect to 100 g of tissue.

Influence of temperature on the amount of acidic and basic groups in tissue. Twenty-five mg of the ground tissue was weighed in the hollow of a "hanging drop" slide. A rubber ring was placed on the slide around the hollow and a second "hanging drop" slide was placed on top with the concave surfaces of the slides face to face so that the tissue could not attach to the upper slide. Then the slides were sealed by plastic tapes and pressed together by 2 screw clamps. No water from the water bath could get inside the "chamber" and the heat was conducted from bath to tissue very quickly. The chamber was placed in a water bath at the desired temperature for 5 min and then removed to ice for some minutes. After opening the chamber, the tissue with the released juice was rinsed from the lower slide into the shaking-bottle for the determination of dye binding. Two ml of buffer was used for rinsing.

Acidic and basic groups were determined by the dye-binding method of Fraenkel-Conrat *(22)*. The sample (25 mg) was shaken at + 2° C for 18 hr with 2 ml buffer and 8 ml dye solution. The mixture was then centrifuged and the supernatant solution diluted.

For determining acidic groups, 0.2% safranine (N.A.D., cert) and phosphate buffer of pH 11.5 were used. The supernatant solution was diluted 250 times and the optical density read at 520 mmc in a 1.0 cm cell. For basic groups 0.1% orange G (N.A.D., cert) and citrate buffer pH 2.2 were used. The dilution was 50 times and the optical density was read at 490 mmc in a 1.0 cm cell. The amounts of dye were sufficient because an increase of the amount of dye did not cause an increase of dye bound by the tissue. The time of shaking also was sufficient because after 24 hr the same amount of dye was bound as after 14 hr.

Amount of dye bound was calculated by means of calibration curves, as follows:

$$\text{Equivalents of acidic groups per } 10^4 \text{ g protein} = \frac{m_s \cdot 28.2}{n}$$

$$\text{Equivalents of basic groups per } 10^4 \text{ g protein} = \frac{m_o \cdot 44.25}{n}$$

m_s = mg bound safranine
m_o = mg bound orange G
n = mg protein per 25 mg tissue.

To determine the protein content of meat, 1 g of ground muscle tissue was soaked in 10 ml distilled water for 30 min and then 20 ml 15% TCA was added. After keeping in the refrigerator for 60 min with occasional stirring, the supernatant liquid was decanted and centrifuged. The precipitate was washed twice with 10 ml distilled water. The nitrogen in the precipitate was determined by the Kjeldahl method.

Influence of temperature on the solubility of globular and structural muscle proteins. A large test tube with 20 g of ground metat was closed by a stopper with capillary, placed in a water bath of the desired temperature for 30 min, and finally cooled in ice water for about 15 min. Twenty g of the ground fresh meat or the whole amount of heated meat including the released juice was extracted 3 times by 0.1 M KCl, according to Hasselbach's *(37)* method, for globular proteins. Another 20 g sample of the fresh or the heated meat was extracted 3 times by stirring with phosphate buffer (pH 6.30, μ 0.64, containing 0.1 M $K_4P_2O_7$) and then 2 times with 0.6 M KCl in a blender, according to the methods of Hasselbach and Schneider *(38)*. Protein denaturations in the serial extracts showed that the 3 phosphate buffer extractions and also the two 0.6 M KCl extractions extracted the soluble structural proteins almost completely.

Total nitrogen in 1 g samples of tissue or 5 ml samples of extracts was determined by the Kjeldahl method. In determining the protein-nitrogen in the extracts 15 ml of 15% TCA was added to 5 ml of the extract. After 30 min it was filtered into a Kjeldahl flask and washed with 3% TCA. The filtrate plus washing fluid was digested and nitrogen was determined using the micro-Kjeldahl method.

To determine the viscosity in protein extracts, relative viscosity (η_{rel}) was measured by means of an Ostwald viscosimeter at 20° C.

$$\text{Viscosity number } Z_\eta = \frac{2.303 \cdot \log \eta_{rel}}{c}$$

c = g protein per 1000 ml extract

ATP-sensitivity was determined by the method of Hasselbach and Schneider *(38)*.

Moisture and pH. To determine moisture, about 1 g of meat was heated in an oven at 105° C for 6 hr. The pH was measured and the electrometric titrations were made by means of a Beckman pH Meter Model G.

RESULTS AND DISCUSSION

Influence of pH on the hydration of fresh and heated meat. The determination of the effect of pH on the water-holding capacity of meat, measured by the press method, makes it possible to find the isoelectric point (I.P.) of tissue (minimum of hydration) and certain relative changes of charges which are available for binding water dipoles. The interpretation

of pH-hydration-curves with regard to the I.P. and to the negaitve and positive charges of the muscle proteins was presented in a previous paper *(34)*.

Study of the influence of mild heating (40° C) on the pH-hydration-curve of meat, which had been adjusted to different pH values after heating, showed no significant shift of I.P., but the water-holding capacity decreased markedly (Fig. 1). Minimum hydration is near the I.P. of muscle (pH 5.5).

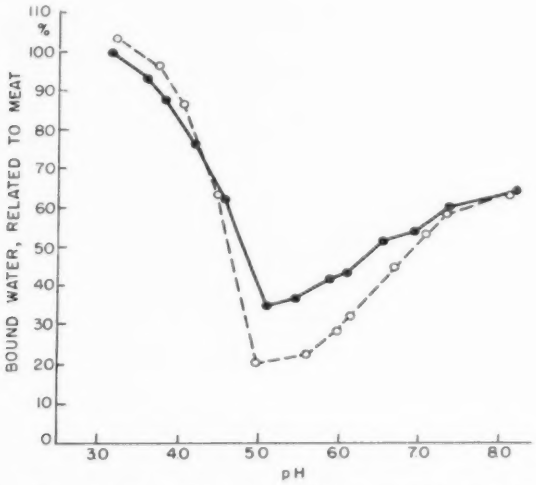


Figure 1. Influence of pH on the water holding capacity of fresh (●, 20° C) and mildly heated (○, 40° C) meat.

It is interesting that the muscle did not lose its ability to hold water at pH values $\leqq$4.5 and $\geqq$7.0. This means that during heating up to 40° C the dehydration cannot be caused by the formation of stable cross linkages between the peptide chains as disulfide or peptide bridges. It is probable that the dehydration and the shrinking of tissue in this range of temperature are produced by a certain unfolding of peptide chains. These changes may cause the formation of salt or hydrogen bonds around the I.P. of muscle. Less polar groups of protein are available for binding water in this range of pH because of steric effects. By adding acid or base these linkages shift or disappear and the hydration is restored. The higher hydration of the heated meat at pH values $\leqq$3 could be explained by an unfolding of peptide chains which will increase the amount of available protein charges in the range of this pH. Similar results were obtained from the muscles of 2 additional animals.

Studies of the influence of higher temperatures (45°, 60°, 80° C) on the pH-hydration-curve of muscle tissue show that heating from 40 to 45° C causes a further decrease of meat hydration (Fig. 2). In this range of temperature a sharp drop of water-holding capacity occurs in the basic range of I.P., but, at pH values <4.5, the hydration increases.

The decrease of the water holding capacity at pH >I.P. and the increase at pH <I.P. suggest a disappearance of acidic groups on heating at 45° C. The decrease of negative protein charges results at pH values >I.P. in a decrease of the electrostatic repulsion between the peptide chains, causing a tighter network of protein structure and a lower water holding capacity:

$$R{-}COO^- \qquad {}^-OOC{-}R' \rightarrow R{-}COO^- \quad R'$$

At pH values <I.P., a decrease of negative protein charges will disrupt salt cross linkages, resulting in a loosening of protein structure and, consequently, in an increase of water holding capacity.

$$R{-}COO^- \cdots {}^+NH_3{-}R' \rightarrow R \qquad {}^+NH_3{-}R'$$

The decrease of carboxyl groups explains the marked shift of I.P. to higher pH values in this range of temperature. By the decrease of acidic groups the muscle proteins become more basic and, therefore, the I.P. is shifted to a more basic pH value. Such a shift of I.P. by heating has been observed with other proteins *(2, 64)*.

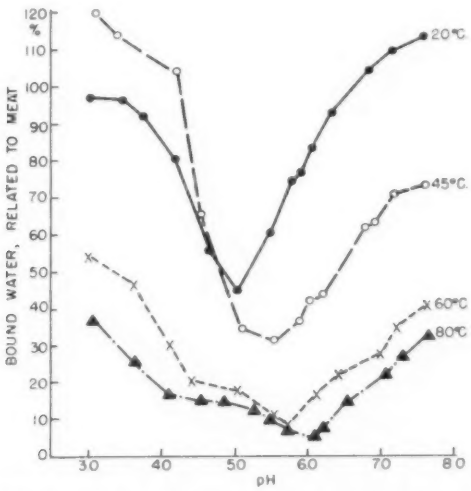


Figure 2. Influence of pH on the water holding capacity of meat after heating to different temperatures.

There seems to be no dehydration and shrinking by formation of new stable cross linkages at 45° C because at pH values <5.0 no decrease of hydration was observed. Disulfide or peptide bonds probably could not have been split in this weak acidic range.

Between 45° and 80° C the decrease of acidic groups seems to continue because of the shift of I.P. to higher pH values, with rising temperature. An increase of temperature from 45° to 60° C results in a marked loss of water-holding capacity at all pH values, indicating a drop of available polar

groups and probably the formation of new stable cross linkages. These linkages may cause a tighter network of protein structure. They cannot be split by raising or lowering the pH in the range between 3.0 and 8.0.

Heating meat from 60 to 80° C causes only small changes in muscle hydration. There is a further decrease of available polar groups (decrease of water-holding capacity at high and low pH values).

When meat is heated from 20° to 80° C, a greater decrease of muscle hydration is observed at high pH values (7.5) than at low values (3.0). This means that in meat heated at 80° C the relative proportion of basic to acidic groups is greater than in fresh meat.

In these experiments the pH of meat was changed by adding acid or base. Proteins can be denatured by acids or bases, but, according to Cannan *(10)* and Mirsky *(49)*, at pH 3.0 and 7.0 denaturation is of minor importance. This also seems to be confirmed by the fact that adding base or acid did not change the pH after a period of time even at pH 3.0 and 7.5.

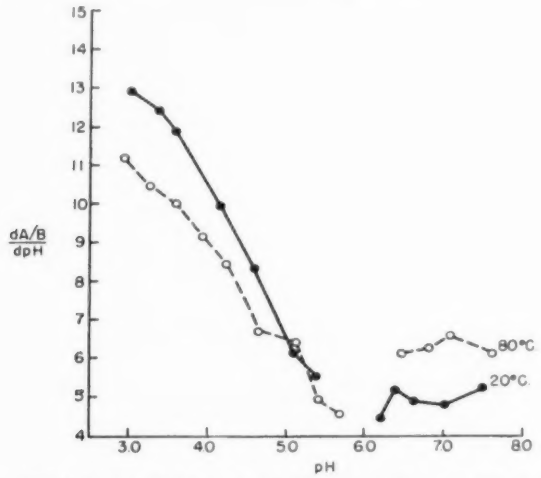


Figure 3. The influence of pH on the buffer power of fresh and heated meat.

Influence of pH on the buffer power of fresh and heated meat. The buffer capacity of muscle and of tissue extracts can be determined by electrometric titration. In this report the titration curves are not shown, but the figures of buffer power are calculated from these curves. The buffer power is defined by the term dA/dpH or dB/dpH. A means meq added acid; B meq added base. Change of buffer power of muscle post mortem and participation of the single components of muscle (structural proteins, water soluble proteins, water soluble non-proteins) in the buffer power of the total tissue were reported previously *(35)*.

In 2 experiments the influence of heating on the buffer power of tissue at different pH values was examined (Fig. 3). In both examples an increase of buffer power was found at high pH values (pH>6.5) and a decrease at low pH values. Decrease of buffer power at low pH can be explained by

the decrease of acidic groups (p_K of $-COOH$ groups: 3.0–4.7) by heating (see above). Consequently, buffer power in the basic range of I.P. will increase because the protein becomes more basic by disappearance of acidic groups. The p_K values of the basic groups in proteins range from 5.6 to 12.6.

It would be conceivable that the higher buffer power of heated meat at about pH 7 is due not to any changes of the proteins but to a release of buffering, protein-bound phosphoric acid compounds. To answer this question, the buffer capacity of the trichloracetic acid (TCA) extracts of fresh and heated (80° C) meat was determined (Fig 4.). The buffer capacity of

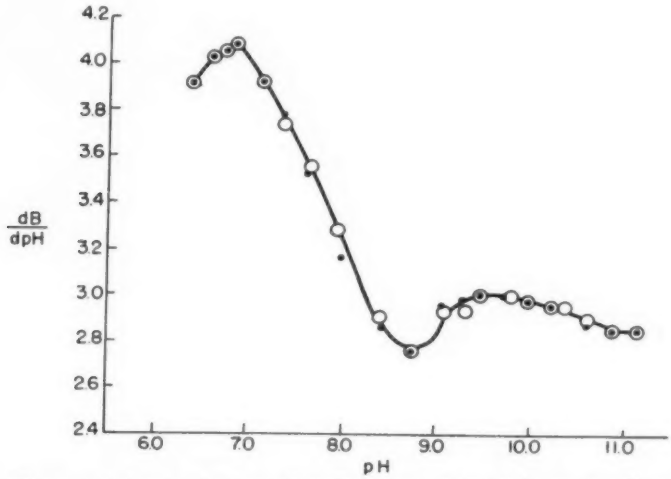


Figure 4. Influence of pH on the buffer power of the trichloracetic acid extracts of fresh (●, 20° C) and heated (●, 80° C) meat.

both extracts are identical. Therefore, no substances buffering in this range of pH (phosphoric acid, nucleotides, carnosine, amino acids, peptides) are released by heating. Bendall *(6)* also found that the proportion of the different fractions of phosphoric acid compounds in muscle is not changed by heating.

Therefore, the observed changes of buffer capacity by heating are indeed due to changes in the muscle proteins. Many investigators have not observed any shift of titration curves by heating other proteins, but Bull *(9)* found that some destruction of the zwitterionic structure accompanies protein denaturation.

Heating influences the buffer power of the water extracts of tissue; in the pH range $\geqq 6$ heat coagulation of water-soluble proteins changes the buffer power of the extract from a phosphoric acid-protein-type (high buffer capacity at pH 7 and pH 11) to a curve almost the same as that of the TCA extracts (Fig. 5).

In the acidic range the buffer power of the water extract of heated meat is lower than that of fresh meat owing to heat coagulation of water-soluble proteins (Fig. 6). The slope of the buffer curve of the 80°-meat extract at

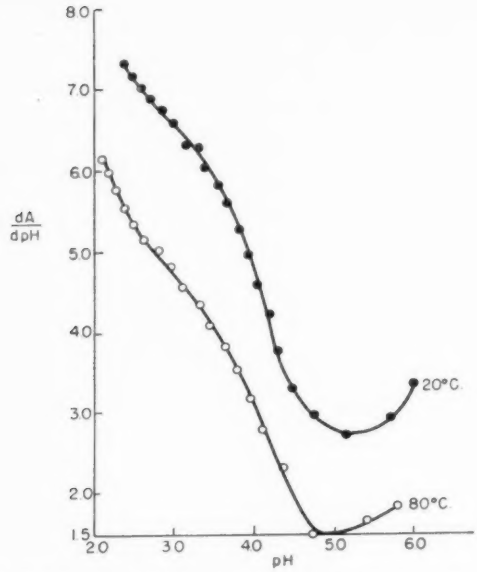


Figure 5. The influence of pH on the buffer power of water extracts of fresh and heated meat; pH $\leqq$ 6.0.

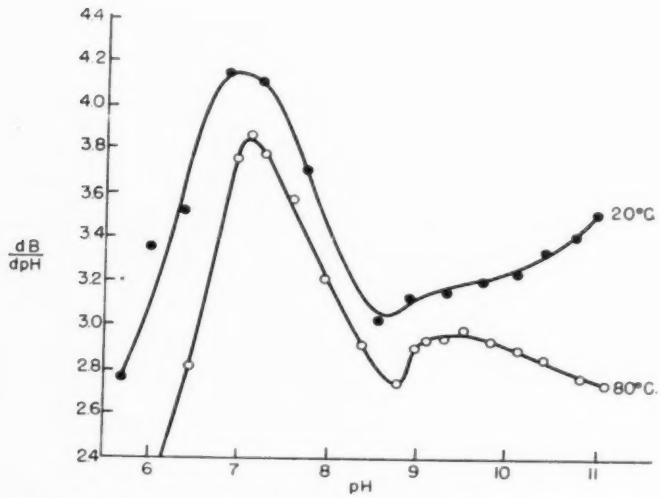


Figure 6. The influence of pH on the buffer power of water extracts of fresh and heated meat; pH $\geqq$ 6.0.

pH values <4 shows that lactic acid (p_K' 3.90) is not solely responsible for the buffering in the acidic range. Probably there are other buffering substances with low p_K values as amino acids or peptides.

Influence of temperature on the hydration and rigidity of meat at two different pH values, adjusted after heating. The results above indicate that heat denaturation of meat occurs in different steps. The following experiments were conducted to define the temperature of these changes more precisely. Meat was heated at different temperatures and then the pH of the heated tissue, including the released juice, was adjusted to pH 5.5 and to pH 3.0 (Fig. 7).

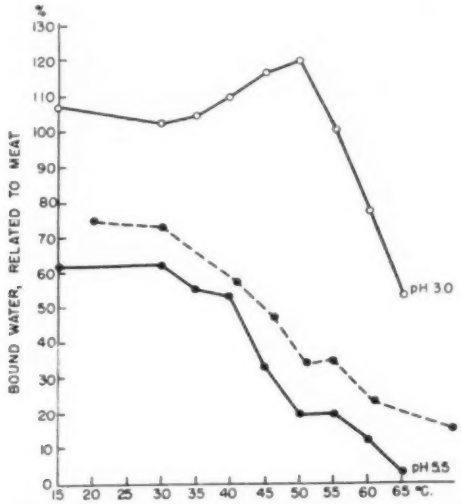


Figure 7. Influence of temperature on the hydration of meat. Meat heated at its normal pH of 5.5 and then adjusted to pH 5.5 (●); meat heated at its normal pH of 5.5 and then adjusted to pH 3.0 (○); and meat heated at its normal pH of 5.5 but not adjusted after heating (● - - - ●).

At pH 5.5, between 20 and 30° C, no changes occur. A decrease of hydration starts at 30° C but the change is not very great until 40° C. The greatest decrease of water-holding capacity lies between 40° and 50° C. Between 50 and 55° C a marked retardation of the decrease of hydration occurs. In this range of temperature there is no drop of hydration, but sometimes a little increase. From 60° C the hydration decreases continuously with increasing temperature, but the decrease is less pronounced than between 40 and 50° C.

Meat heated at its normal pH (5.5) with no change of pH after heating shows the same step of hydration between 50 and 55° C. Wierbicki and Deatherage *(68)* also observed such a step with quite another method when they heated meat at its normal pH value.

In the acidic range of I.P. (pH 3.0) almost no change of hydration occurs between 20 and 30° C. Hydration increases between 30 and 35° C

and reaches a sharp maximum at 50° C. From 55° C the hydration drops continuously with increasing temperature. According to these results, the formation of new stable cross linkages which cannot be disrupted in the acidic range of I.P. starts between 50 and 55° C.

Change in the hydration curve at pH 5.5 occurs at about the same temperature at which the meat of pH 3.0 shows a peak of hydration. There seems to be no direct connection between these phenomena because between 50 and 55° C the decrease of meat hydration at pH 5.5 stops but the decrease of hydration at pH 3.0 continues. Therefore, the step between 50 and 55° C at pH 5.5 is caused neither by a relative increase of basic groups nor by a delay of the formation of new cross linkages. Consequently, the step seems to be caused by the fact that no acidic groups have yet disappeared. According to the theory that a denaturation is accompanied by an unfolding of peptide chains, an increase of hydration would be expected. But many authors point out that during the denaturation of different proteins a dehydration can be shown and that this effect is caused by a reorientation of protein structure, including new polar or nonpolar cross linkages *(9, 40, 42, 57)*. The fact that dehydrated fresh muscle tissue absorbs water faster than dehydrated cooked muscle *(63)* may be explained in this way.

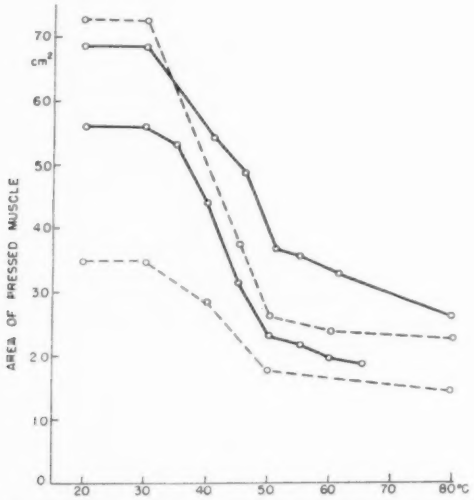


Figure 8. Influence of temperature on the rigidity (area of pressed muscle) of four different meats.

The "press method" for determining hydration permits an estimation of the rigidity of tissue by measurement of the area of the pressed muscle sample *(29)*. Figure 8 shows that in the *longissimus dorsi* muscles of 4 different animals the increase of rigidity by heating at pH 5.5 corresponds to the decrease of hydration (decreasing meat area is related to the increasing rigidity). The "coagulation" starts at about 35° C, similar to pure actomyosin *(25, 45)*. The "coagulation" is almost finished at 55° C. Between

55 and 80° C the decrease of hydration seems to be stronger than the increase of rigidity. Those curves also show a delay of the increase of rigidity and this corresponds to the step of the hydration curves. Our results agree with those of Lobanov and Bykova *(46)* who found that denaturation of beef starts at above 30° C.

Influence of the pH of meat before heating on the change of water-holding capacity after heating. With regard to cooking and processing of meat, it is important to know if the influence of pH on the water-holding capacity of muscle is retained after heat denaturation. It is conceivable that a strong denaturation, as caused by heating at 80° C, might supersede any pH effects.

Raw meat was adjusted to various pH values and heated to 80° C. Figure 9 shows that the pH of meat has a marked influence on the water-holding capacity, measured by the press method, not only before but also after heating. Cooked meat with the lowest water-holding capacity is meat which is heated at its I.P. (about pH 5.0). By heating meats with higher and lower pH values, the resulting cooked meats have a better water-holding capacity; the hydration increases with increasing or decreasing pH.

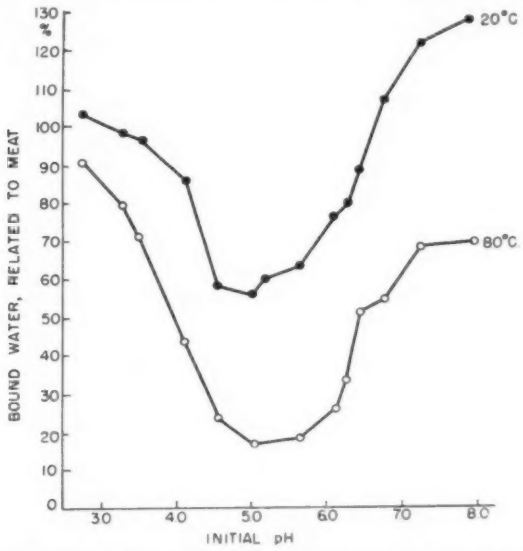


Figure 9. Influence of pH of meat before heating on the change of water holding capacity after heating to 80° C.

Indeed, the pH-hydration curve of cooked meat is quite similar to the corresponding curve for the raw muscle. Our experimental results show considerable differences between the heating in the acidic range and heating in the basic range of I.P. Heating of meat at low pH values results in a lesser drop of hydration than heating at higher pH. These results were repeated on several different meats.

It was established previously that heating causes a loss of acidic groups

in the muscle proteins. Therefore, it is understandable that heating at low pH, at which the muscle has more positively charged groups than negative groups, causes less drop of hydration than heating at higher pH values. At low pH the decrease of the net charge of proteins is less than at high pH.

According to these results, it is presumed that carboxyl groups are more important for the formation of a tighter network of protein structure during denaturation than basic groups. Other factors may also play a role. Warner and Levy *(65)* found that the rate of disappearance of sulfhydryl groups by heating bovine plasma albumin increases from pH 3 to pH 8. Therefore, it could be that the dehydration of muscle by heating at lower pH values is less than at higher pH where thiol groups may form stable disulfide or thioether cross linkages. This problem is being investigated.

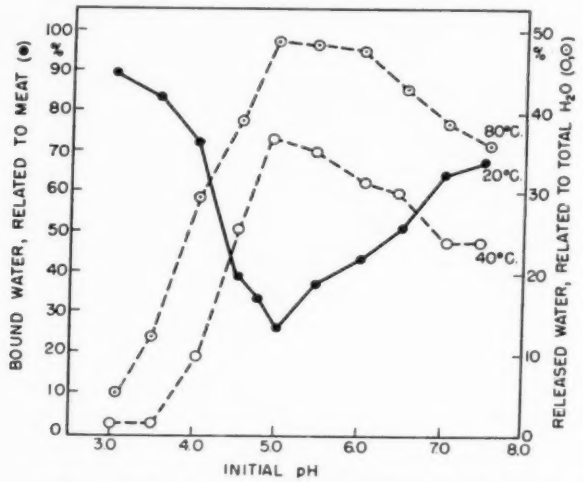


Figure 10. Influence of pH of meat before heating on the release of juice by heating at 40° and 80° C.

The relative differences of the water-holding capacity of raw muscle at different pH values are retained after heating. Bendall *(6)* also found that meat heated at high pH has a higher water-holding capacity than meat heated at its normal pH. Therefore, the network of new cross linkages, formed by heat denaturation, cannot completely overcome an increase of hydration caused by the lowering or rising of the pH of raw meat. Apparently the influence of pH on denaturation of meat is related to the unique steric conditions of the proteins in the muscle fiber and not by the normal influence of pH on the kinetics of denaturation since it has been observed with other proteins that the rate of denaturation has a minimum at the I.P. *(13, 14, 44, 65)*.

Influence of the pH of meat before heating on the release of water by heating. In processing or cooking meat, a loss of juice is caused by denaturation. In order to study the influence of pH before heating on this effect, it was necessary to use a method without pressing or centrifuging.

For this reason water content of the juice released by heating was measured. The result of this experiment (Fig. 10) agrees with the results obtained by the press method. Water released by heating of muscle is maximal at its I.P. and reflects the state of hydration before heating. Of course, more water is released at 80° C than at 40° C, but the relative differences with respect to pH are quite similar. These results are not surprising, because investigations on the aging of meat *(32, 66, 67)* and on the influence of salts *(33, 36, 68)* have shown that the release of juice by heating is inversely proportional to the hydration of the raw meat.

Table 1 shows that the content of dry matter in the juice released by heating at 80° C has a minimum at the I.P. of muscle. Therefore, the greater release of juice around the I.P. does not have too much effect on the percent of solid matter in the expressed juice.

TABLE 1

Influence of the pH of Meat Before Heating on the Amount of Dry Matter in Juice Released by Heating at 80° C.

pH before heating	Dry matter in juice %
7.56	4.60
7.05	4.43
6.53	4.03
6.00	3.60
5.45	3.54
4.98	3.53
4.54	3.69
4.05	3.91
3.50	4.47
3.04	4.96

Influence of heating on the pH of meat. It is known that heating proteins in solution results in an increase of pH *(3, 9, 64)*, and this has also been observed on meat *(6, 53, 68)*. Bendall *(6)* found that this increase even becomes greater if the water soluble buffering substances of meat are removed. This change of pH apparently represents changes of protein charge. The decrease of acidic groups which shifts the I.P. to higher pH values may explain the rise of pH by heating of muscle at its normal pH. In order to get more detailed information concerning this effect, the influence of pH and temperature on the shift of pH by heating was studied.

Figure 11 shows the influence of the pH of meat before heating on the shift of pH by heating at 80° C. In 4 samples representing different animals a sharp maximum of the pH-change (ΔpH) was observed in the range of the I.P. When data on 60 different meat samples ranging from pH 4.0 to 7.0 were examined, the shift of pH by denaturation at 80° C was found to be at a maximum increase in pH between 5.2 and 5.6. Bendall *(6)* and Wierbicki *et al (67)* observed that the ΔpH produced by heating decreases with increasing pH in the basic range of I.P. Under experimental conditions in this laboratory, negative ΔpH values were never found at a pH before heating >5.0, but at pH values <5.0 negative ΔpH values were sometimes observed.

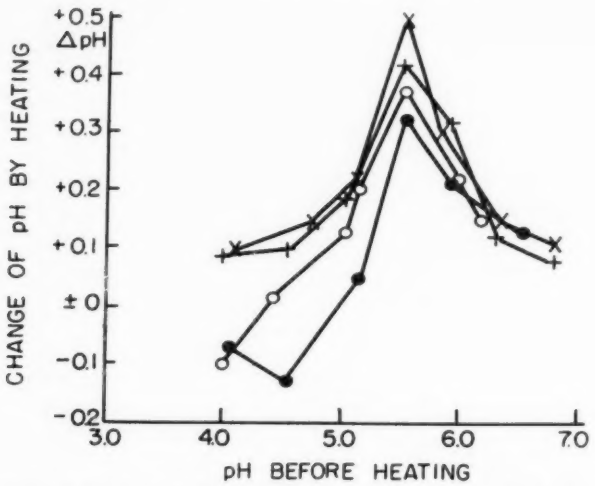


Figure 11. The effect of pH before heating on the change of pH (Δ pH) produced by heating four different meats to 80° C.

The reason for the maximum of ΔpH on heating may be the loss of acidic groups. At higher pH values the dissociation of acidic groups increases and that of basic groups decreases. As more positive charges on the proteins are dissipated by increasing the pH, the disappearance of negative groups by denaturation will have proportionally less influence on the pH. On the other hand, with lower pH values the dissociation of basic groups increases and the dissociation of acidic groups decreases and, as more negative groups

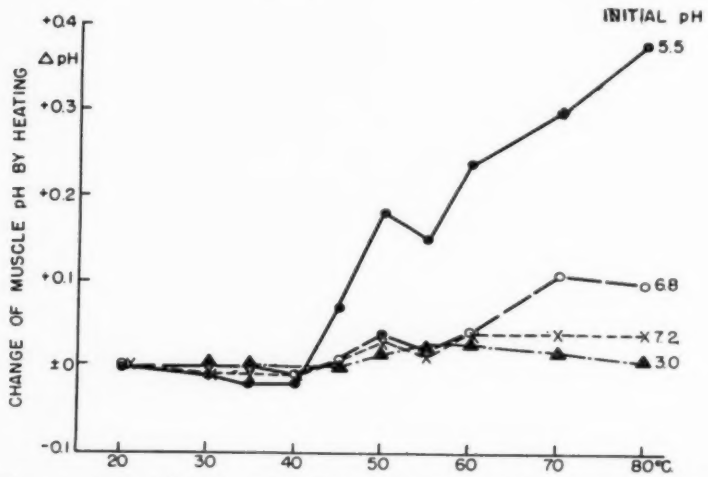


Figure 12. The relationship of initial pH and temperature of heating to the change of pH produced by heating meat.

are discharged, the decrease of negative groups by heating will have less influence.

The absolute values of the pH shift by heating are different for different meats. The ΔpH differences are greater on the acidic side than on the basic side (Fig. 11). According to preliminary experiments, these differences seem to be caused by the fact that variations in negative groups for meats are greater than those of positive groups. Further work is necessary to determine whether this correlation is significant.

Figure 12 shows the relationship of temperature of heating on the change of pH for meats of different initial pH values. At the normal pH of meat (5.5), a significant change of pH does not occur until 40° C. Between 40 and 50° C the pH increases quite markedly. After reaching a maximum at 50° C, the pH drops a little between 50 and 55° C. At temperatures >55° C the pH increases with increasing temperature.

The change of ΔpH with temperature corresponds exactly to the change of meat hydration with temperature. Marked changes start at 40° C and the significant step lies between 50 and 55° C. The interpretation of the change of pH by heating agrees with interpretation of the change of hydration by heating. Both are caused by the decrease of acidic groups.

At an initial pH of 6.8, increasing temperature has a slight influence on the pH of meat and, at an initial pH of 7.2, it has almost no influence. In both cases it should be noted that a minor peak of pH occurs at 50° C. Heating meat at pH 3.0 has no significant influence on the pH at each temperature between 20 and 80° C.

It has been suggested that the increase of pH by heating would have a favorable influence on the water-holding capacity; yet this appears to be offset somewhat by the fact that at the same time the minimum of hydration is shifted to higher pH values.

Influence of temperature on the amount of acidic and basic groups in meat. The method of determining basic and acidic groups in meat proteins by binding of dyes that we used concerns both water soluble and insoluble proteins. The soluble proteins are precipitated by the dye. The dye binding was determined only at very high (pH 11.5) and very low (2.2) pH values. Normally at these pH values almost all acidic groups or all basic groups are dissociated and available for binding dyes. Changes in protein charges and in protein structure which concern only a change of protein net charge and the formation of salt cross linkages around the I.P. will not influence dye binding at this high or low pH value. Therefore, any changes in dye binding at these pH values are due to reactions of these groups resulting in new covalent bonds such as peptide or ester bonds or perhaps also in strong complex bonds (e.g., with metals). We found for untreated aged beef muscle 15.1 equivalents of acidic groups per 10^4 g protein (st. dev. 0.8) and 13.2 equivalents of basic groups (st. dev. 0.2). These values agree favorably with reports that L-myosin contains 16.5 equivalents acidic and 15.5 equivalents basic groups per 10^4 g protein *(18, 47, 48)*. Edsall *(17)* points out that myosin contains more polar groups than most other proteins. Our figures are a little lower than those of L-myosin, and this may be due to the facts that (a) proteins other than myosin (e.g., actin, globular proteins) are pres-

ent in muscle and contain fewer charged groups (tropomyosin is similar to myosin *(17)*), and/or (b) in the muscle fiber fewer charged groups are present or available than in the solute myosin.

No change of acidic groups occurs between 20° C and 40° C, but between 40 and 50° C there is a decrease (Fig. 13). Between 50° C and 55° C the number of these groups is not changed. Between 55 and 60° C they decrease very markedly. Loss of acidic groups is continued between 60 and 70° C but not so pronounced as between 55° C and 60° C. This curve was reproducible on another sample. By heating from 20° C up to 70° C, muscle loses about one third of its carboxyl groups.

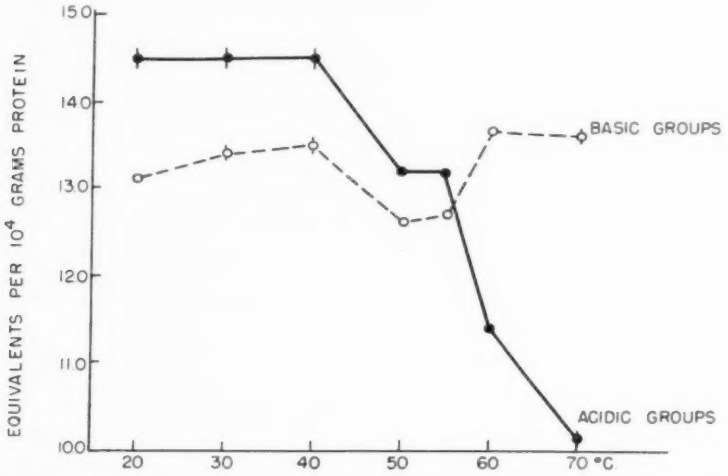


Figure 13. The influence of temperature on the numbers of basic and acidic groups in meat.

This curve with all its marked features as the start of changes at 40° C and the step between 50 and 55° C corresponds exactly to the curve of the decrease of hydration and of the increase of pH by heating of muscle at its normal pH. This agreement is evidence that the characteristic changes of hydration and of pH are caused by a stepwise decrease of carboxyl groups. As to the basic groups of proteins, there is no significant decrease by heating in basic groups of muscle protein during heating from 20° C to 70° C. The increase in the range from 20° C to 40° C is not significant because it was not found in another sample. Although the slight minimum of basic groups at about 50° C was observed in the second sample, it was much weaker. These little or no changes in basic groups during heating agree with the results of the hydration and pH-experiments described above.

No changes in the charges on protein have also been observed between 20 and 40° C by other investigators *(8, 15, 41, 42, 44, 59, 69)*, but no evidence for any loss of acidic groups at higher temperatures is reported. According to Mirsky *(51)* and Mirsky and Pauling *(52)*, an increase of pH

and a shift of the I.P. to higher pH values by denaturation may be theoretically explained by a cleavage of salt type cross linkages; but, according to data reported here, in the case of muscle proteins *in situ* the increase of pH and the shift of I.P. must be caused by a decrease of carboxyl groups because the change in pH corresponds exactly to the decrease of acidic groups.

Influence of heating on the solubility of globular and structural proteins of muscle. It is of interest to compare the influence of heating on hydration and rigidity of tissue with the change in the solubility of muscle proteins. At first the influence of temperature on the solubility of the globular muscle proteins at low ionic strength was studied. At 20° C, about 25% of the total proteins of aged meat are extractable with 0.1 M KCl. This fraction may consist of myogens, myoglobin, some enzymes, and globulin X. The amount of globular proteins we found is somewhat lower than that which Hasselbach and Schneider *(38)* extracted from the rabbit muscle immediately post mortem *(28%)*.

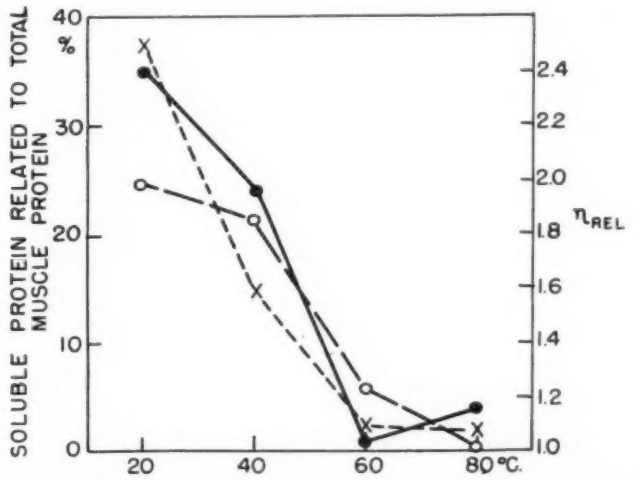


Figure 14. The effect of heating on the structural protein, (●), globular protein (○), and viscosity of phosphate extracts (x) of beef muscle.

With increasing temperature the solubility of this protein fraction decreases (Fig. 14). Between 20° C and 40° C the change of solubility by heating is relatively small but between 40 and 60° C solubility decreases markedly. After heating at 60° C only 23% of the proteins soluble at 20° C are extracted. Between 60 and 80° C the solubility decreases only slowly with increasing temperature. At 80° C, less than 1% of the proteins soluble at 20% C is extractable.

Hasselbach and Schneider *(38)* could extract from the rabbit muscle immediately after death pure myosin by phosphate buffer of μ 0.6 and pH 6.3 in the presence of pyrophosphate and the pure actin after extraction of

myosin and mincing in a blender by 0.6 M KCl. We found that it is not possible to get a complete separation of myosin and actin if the aged meat is extracted by this method. According to the ATP-sensitivity, the "myosin"-extract (phosphate buffer) contains some actin and the "actin"-extract (0.6 M KCl) contains myosin. The sum of the proteins of both extracts minus the dissolved globular proteins will be called the "soluble structural proteins." Hasselbach and Schneider *(38)* extracted about 50% actin plus myosin (related to total protein) from the rabbit muscle immediately after slaughter. We found for aged beef muscle only 35% actin plus myosin. It is to be expected that the structural proteins in fresh muscle are more soluble than in aged muscle *(32, 67)*.

By heating from 20 to 40° C a marked decrease of the solubility of structural proteins occurs, but the greatest decrease is observed by heating from 40 to 60° C. Beyond 60° C the structural proteins become almost insoluble. After heating at 80° C, a small amount of soluble proteins (4% of total protein) was found, but it is not certain that this increase of solubility is significant. It may be noted that Bourdillon *(7)* could isolate a heat stable, crystalline protein after heating of muscle at 90° C.

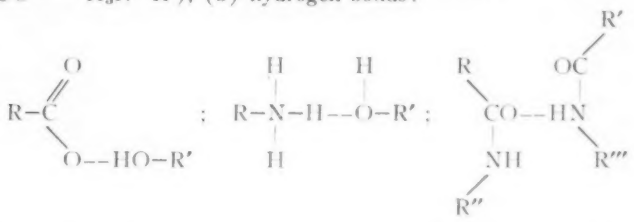
At 40° C, the structural proteins seem to be more sensitive than the globular proteins. By heating from 20 to 40° C, the viscosity number (Z_η) of the proteins soluble in phosphate buffer decreases from 0.103 to 0.069 and also the ATP sensitivity decreases from 20 to 13. Therefore, it appears that the structural proteins which are still soluble at 40° C are changed by heating.

A slight increase of soluble non-protein-N was observed with increasing temperature. This nitrogen fraction increases by heating from 20 to 80° C from 74.21 to 79.51 mg N per 20 g tissue in the 0.1 M KCl extract and from 80.1 to 87.6 mg N per 20 g tissue in the phosphate buffer extract. It may be possible that at higher temperature, smaller nitrogen containing molecules (e.g., nucleotides, ammonia) bound by proteins are split off.

Results of these solubility-experiments agree quite well with those obtained by measurement of water-holding capacity and rigidity described above. Decrease of muscle hydration and increase of rigidity as well as decrease of protein solubility show that the strongest denaturation of the muscle proteins occurs between 40 and 80° C. But there are also some differences. The decrease of the solubility of structural proteins by heating at 40° C is more pronounced than the drop of hydration. On the other hand, by heating from 60 to 80° C we observed a remarkable decrease of hydration but no further decrease of the solubility of structural proteins, which are almost insoluble at 60° C. Therefore, the process that causes changes in protein hydration cannot be completely identified with the process which causes changes in solubility.

Denaturation is defined as a change in the configuration of the native protein without modification of covalently bound structure *(61, 62)*. This definition would include also any effects of salts on proteins which Putnam *(57, 58)* does not consider as a real denaturation. Denaturation is accompanied by the cleavage of cross linkages within the native protein and in certain cases also by formation of new cross linkages *(1, 39, 42, 55, 57)*. The types of cross linkages that are possible between the peptide chains or also within

one peptide chain are characterized as (a) electrovalent or salt-linkages ($R-COO^- - -{}^+H_3N-R'$), (b) hydrogen bonds:



and (c) covalent linkages: R–S–S–R′; R–CO–NH–R; R–CO–O–R′ etc.

Our work shows that heat denaturation of meat occurs in different steps. According to the hypothesis of Wu *(69)*, denaturation of proteins includes 2 steps. The first reaction is an unfolding of the peptide chains that are folded in the native protein. The second is an aggregation of the chains that finally results in a coagulation of the protein. The unfolding of peptide chains explains the observation that certain groups in the protein, such as thiol groups, inactive in the native protein, become active by denaturation *(26, 57)*. Stauff *(61)*, however, suggests the possibility that the first step is an intramolecular swelling and a loosening of protein structure rather than a real unfolding *(16)*. Usually the first step of denaturation is considered to be reversible, the second, irreversible *(19)*.

Summation. Based on the results reported here and with the background supplied by others cited, it may be well to summarize the steps of meat denaturation by heating.

20 to 30° C. No change in hydration, rigidity, buffer capacity solubility and charges on proteins was observed in this range of temperature.

30 to 40° C. In this temperature range some changes in muscle proteins occur and these influence hydration, rigidity, solubility *(5)* and also the ATPase activity of muscle-actomyosin *(20, 54, 56)*. It may be noted that the proteins in the protein-complex of the intact muscle tissue are much more resistant to heat than in isolated state *(20)*. No marked changes of acidic or basic groups occur between 30 and 40° C. This result agrees with the behavior of the charged groups of other proteins at these temperatures *(46)*. A certain increase of hydration in the acidic range of I.P. may be due to an increase of available charged groups by unfolding of peptide chains. There is some evidence that heat denaturation increases the availability of charged groups in proteins *(3, 9, 57)*.

Changes in muscle proteins between 30 and 40° C include 2 steps: an unfolding of peptide chains and the formation of new electrostatic and/or hydrogen cross linkages. The cause of the formation of such new cross linkages may be the rearrangement of protein structure by unfolding of the peptide chains resulting in a tighter network of protein structure in the isoelectric range. We may compare this reaction with the "second reaction" of denaturation suggested by Bull *(9)*. According to this theory, "flocculation" of denatured molecules occurs preparatory to coagulation. This reaction occurs if the clear sol of the heat denatured protein is brought to the I.P.

The "flocculation" is completely soluble in acid or base in our experiments as the hydration of muscle is completely restored by addition of acid or base.

40 to 50° C. By heating at these temperatures, hydration, rigidity, solubility, pH and I.P. are changed and the acidic groups decrease with increasing temperature. The changes of hydration in this range of temperature are mainly determined by the disappearance of carboxyl groups in the muscle proteins. The reaction causing the drop of carboxyl groups does not appear to form many new cross linkages between the peptide chains because the hydration of meat is not decreased at pH values $\leqq 4.5$. We do not yet know what kind of reaction causes the decrease of the carboxyl groups. There is no reaction with amino groups to form peptide bonds because we did not observe proportional decrease of amino groups. A decarboxylation also is not probable because preliminary experiments show that the amount of CO_2 developed by heating of meat does not correspond to the decrease of carboxyl groups. Other possibilities are an ester linkage R−CO−O−R′, which may be formed with the hydroxy-groups of serine, threonine or tyrosine *(11)* or a thiol ester linkage R−CO−S−R′ *(11)*. A formation of the latter linkage is not very possible because of its lability.

50 to 55° C. In this range of temperature the changes in hydration, rigidity and pH are delayed very markedly and this is perhaps caused by a delay of the decrease of carboxyl groups. At these temperatures the formation of new cross linkages starts; these are quite stable and cannot be split by addition of base or acid in the pH range from 3.0 to 8.0. The formation of new stable cross linkages may represent the "second step" of denaturation according to the theory of Wu *(69)*. The new cross linkages will be mainly disulfide groups *(27, 61)* and perhaps some ester bonds *(11)*. According to our results there is no formation of peptide bonds. Bull *(9)* also points out that it does not seem probable that peptide bond synthesis is a necessary part of coagulation. Of course, at higher temperature formation of additional salt-and hydrogen-bonds may occur *(12)*. Bound cations may also take part in denaturation. Bivalent cations which are bound to the native muscle proteins are released by heating as free ions *(30)*. This release seems to occur in definite steps *(68)* rather than continuously. On the other hand, certain cations, such as Mg^{++} or Mn^{++}, can protect proteins from heat denaturation *(24)*.

Highly hydrated muscle resulting from a high or low pH, or the presence of salts, ATP, etc. is not dehydrated by heating to the same degree as muscle with a lower hydration. Perhaps high hydration renders the reactions forming cross linkages more difficult because of steric hindrance (larger distance between the peptide chains).

As to the decrease in carboxyl groups between 50 and 55° C, several explanations are possible:

(a) Two different kinds of $R-COO^-$-groups could exist which differ in the sensitivity toward heat. Either both are present in all structural muscle proteins or each is located in another protein. In the latter case the different structural muscle proteins (actin, myosin, tropomyosin) would be denatured in a different way.

(b) Two different kinds of reactions are responsible for the decrease of carboxyl groups. The one reaction would be finished at about 50° C, the other would start at about 55° C.

(c) The change of the shape of proteins between 50 and 55° C could present steric conditions which render more difficult the reactions eliminating the free $-COO^-$-groups.

55 to 80° C. Hydration, pH, and the amount of carboxyl groups are changed in this range of temperature but not so markedly as between 40 and 50° C. Both rigidity and solubility changes are very small. In this range of temperature the formation of new cross linkages and the loss of acidic groups is continued. As a temperature of 60-70° C the denaturation of meat seems to be almost complete.

Heating at temperature higher than 80° C. The influence of temperatures higher than 80° C on meat was not investigated by us, but, according to Fraczak and Pajdowski *(21)*, at about 80° C the formation of free H_2S starts and increases with increasing temperature. These changes at higher temperatures are of great practical importance as thermal degradation associated with cooked meat flavors occur.

It should be noted that the changes of hydration and protein charges caused by heat denaturation are completely different from changes which occur during the rigor mortis and the aging of meat *(32, 34, 35, 50, 66, 67, 68)*. Reasons other than protein denaturation are responsible for the post mortem changes of meat.

CONCLUSIONS

Heating of beef from 20 to 30° C results in no remarkable change in muscle proteins. Between 30 and 40° C a mild denaturation occurs, resulting in an unfolding of protein chains and in the formation of new salt and/or hydrogen bonds.

A strong denaturation starts at 40° C and continues to 50° C, resulting in the formation of new stable cross linkages which are not split off by addition of acid or base. The amount of negatively charged groups of the muscle proteins decreases with increasing temperature.

Between 50 and 55° C the denaturation and the formation of new cross linkages are continued, but the decrease of acidic groups is delayed. By heating at temperatures $>55°$ C the negatively charged groups decrease again. The denaturation, causing a tighter network of protein structure, is still continued. At about 65° C the denaturation is almost but not completely finished.

The stepwise change of water-holding capacity of meat and of its pH by heating at its normal pH is determined by the stepwise decrease of the acidic groups in muscle proteins. Heat denaturation does not cause a significant decrease in the amount of basic groups in muscle proteins.

Acknowledgment

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HISTOLOGY AND HISTOCHEMISTRY OF RAW AND COOKED RICE KERNELS[a]

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Most varieties of rice have cooking characteristics peculiar to their grain-length class—long, medium, or short. Cohesiveness, or the tendency of kernels to adhere to each other after cooking, is generally least pronounced in long-grain and most pronounced in short-grain varieties *(1)*. Kernel expansion and disruption also are generally slighter in long-grain than in medium- and short-grain varieties (Figure 1). However, an occasional variety of one grain-type possesses cooking characteristics considered typical of another grain-type, and additional criteria for classification are sometimes necessary. Cohesiveness in general can be linked to known differences in properties of the startch *(3, 8, 13)* but there are also exceptions to this rule. Added information on both starch and non-starch components would increase our understanding of cooking behavior in rice.

The studies reported here were undertaken to augment our basic knowledge of the rice kernel and to explore the possibility that composition and distribution of cell walls, protein and other materials may regulate expansion and disruption of rice kernels when heated in water. The findings were based largely on freehand cross sections of uncooked brown rice kernels and microtome sections of cooked, Carbowax-embedded brown and milled rice, subjected to several standard histochemical treatments, and observed microscopically in bright-field and polarized illumination.

EXPERIMENTAL PROCEDURES

Materials. Of the 23 rice varieties studies histologically (Table 1), all lots but one (Toro) were produced during 1954 at the Rice Pasture Experiment Station, Beaumont, Texas. Two samples were supplied from each lot, one as rough rice and the other milled. The rough rice was husked by hand in the laboratory; samples were stored under refrigeration until used. The milled samples were included in several other studies on rice *(8, 9, 10)*.

Methods. Uncooked brown rice kernels were soaked until completely penetrated either in 10% glycerine, or in a fixative (10% formalin, or modified Newcomer's) containing 25% of propylene glycol as a softening agent, then inserted in a pitch stick and hand-sectioned with a razor blade. Cooked brown and milled kernels, prepared by dropping 1 g rice into 10 ml hot water and holding in a boiling water bath 30 min, were embedded in Carbowax before sectioning *(7)*.

Stains included iodine vapor *(6)*, ferric ferricyanide reagent *(5)*, ruthenium red, erythrosin B, and oil red O. Oil red O was prepared by making a saturated solution of the dye in equal parts of acetone and 70% ethanol with 1% benzoic acid *(4)*, and diluting with an equal volume of additional solvent. Iodine was applied in dilute aqueous solution for photomicrography. Zinc-chloride-iodide reagent *(11)* was used as a test for cellulose.

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[a] Presented at the Nineteenth Annual Meeting of the Institute of Food Technologists, Philadelphia, Pennsylvania, May 17, 1959.

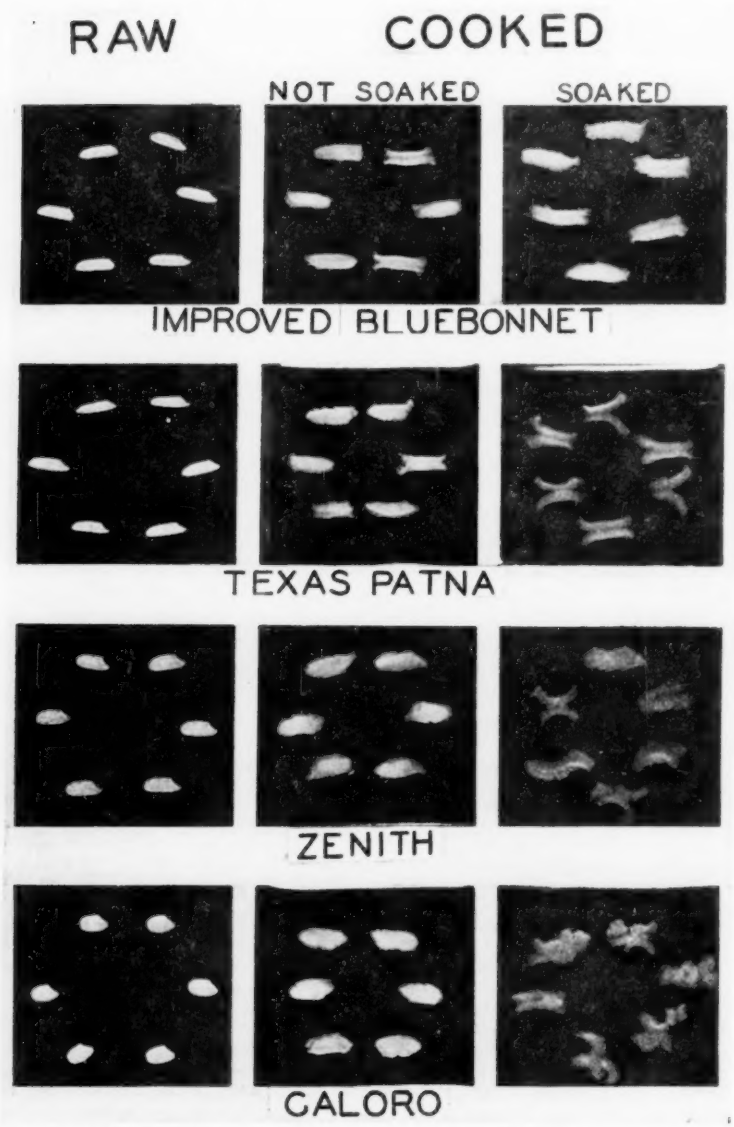

Figure 1. Milled white rice from the varieties Improved Bluebonnet (long-grain), Texas Patna (long-grain), Zenith (medium-grain), and Caloro (short-grain), illustrating differences in appearance before cooking, immediately after cooking, and after simulated overnight soaking to accentuate differences in disruption patterns.

TABLE 1

Average dimensions[1] of cross sections, and of cells in two positions from cross sections of brown rice varieties

Variety and grain length[2]	Sections			Cells					
				Dorsal			Ventral		
	Diameters		Areas	Dimensions		Ratios	Dimensions		Ratios
	D_1	D_2		d_1	d_2		d_1	d_2	
	mm	mm	sq mm	μ/10	μ/10	d_1/d_2	μ/10	μ/10	d_1/d_2
Bluebonnet (L)	2.4	1.8	3.8	15	5	3.0	14	5	2.8
Bluebonnet 50 (L)	2.4	1.8	3.8	15	6	2.5	15	7	2.1
Blue Rose (M)	3.3	2.3	6.0	22	6	3.7	27	5	5.4
Caloro (S)	3.3	2.4	6.2	17	5	3.4	19	5	3.8
Calrose (M)	2.9	2.1	4.8	16	5	3.2	16	5	3.2
Century Patna 231 (L)	2.3	1.6	2.7	14	5	2.8	8	5	1.6
Colusa (S)	3.4	2.2	5.9	20	4	5.0	21	5	4.2
Early Prolific (M)	3.0	2.0	4.7	16	5	3.2	18	4	4.5
Fortuna (L)	2.6	1.8	3.7	16	6	2.7	16	6	2.7
Improved Bluebonnet (L)	2.3	1.8	3.2	14	6	2.3	14	5	2.8
Magnolia (M)	3.0	2.0	4.7	17	6	2.8	17	6	2.8
Nato (M)	3.0	2.0	4.7	15	6	2.5	16	6	2.7
Rexark (L)	2.4	1.8	3.8	16	7	2.3	14	7	2.0
Rexoro (L)	2.4	1.8	3.8	14	4	3.5	14	6	2.3
Sunbonnet (L)	2.2	1.7	2.9	12	6	2.0	15	5	3.0
Texas Patna (L)	2.2	1.7	2.9	13	7	1.8	14	7	2.0
Texas Patna 49 (L)	2.3	1.7	3.1	14	5	2.8	14	5	2.8
Toro (L)	2.5	1.8	3.5	14	6	2.3	12	5	2.4
Zenith (M)	2.8	1.8	4.0	19	5	3.8	18	5	3.6
B4512A1-20(L)	2.2	1.8	3.1	10	6	1.7	12	7	1.7
11-47-11-1 (S)	3.0	2.2	5.2	14	5	2.8	15	5	3.0
B455A1-25 (L)	2.3	1.9	3.4	13	6	2.2	13	5	2.6
12-47-6-2 (S)	3.1	2.1	5.1	17	5	3.4	16	5	3.2

[1] D_1 and D_2 indicate dorsiventral (top to bottom) and lateral (side to side) diameters respectively of cross sections; d_1 and d_2 represent radiating and sectoring dimensions of cells in the area dorsal to the central core and in the area ventral to the central core.

[2] Grain lengths are long (L), medium (M), and short (S).

Many observations and most of the photomicrographs were based on sections mounted in glycerol. With the aid of an ocular micrometer, cross sections representing two kernels of uncooked brown rice of each variety were measured. The long or dorsiventral diameter (D_1), and the short or lateral diameter (D_2), were converted to millimeters, and areas of the cross sections were calculated by the formula $pi \cdot R_1 \cdot R_2$.

Endosperm cells in the small-celled central portion or "core," in the area dorsal to the core, and in the area ventral to the core, also were measured. Two dimensions were recorded for each of 20 cells in each location, one indicating the distance from the more central edge to the more peripheral edge of the cell (the radiating dimension, or d_1), and the other indicating the distance from one edge of the cell to the other at right angles to d_1 (the sectoring dimension, or d_2). For a quantitative expression of cell shape, the ratio of radiating to sectoring dimension was secured by the formula d_1/d_2. Areas of the cells in cross section were calculated for exploratory purposes by the formula $d_1 \cdot d_2$.

OBSERVATIONS

Structure of uncooked rice. A considerable range in size of the cross-sections was observed (Table 1; Figures *2*, 3). As expected, smaller cross sections were usually from long-grain rices, and larger sections usually from medium- and short-grain varieties, with some exceptions. Kernels of large diameter often had a pre-formed dorsiventral rift in the center.

The bran, composed of pericarp, testa and aleurone *(2)*, was thicker along the longitudinal dorsal line or "ridge" than around the lateral and ventral surfaces (Figure 4A). Cell walls of the pericarp and aleurone were about *2* mc thick, and reacted positively to tests for protein (B)[b], for pectin or hemicellulose (C) and for cellulose (D, E). The testa or seed-coat was shown by its reaction with oil red O to contain material of a fatty nature (F), probably suberin which is a deterrent to water penetration; suberization was diffuse or interrupted along the dorsal ridge (G). A sheath of fat-staining material enclosed the aleurone grains.

The starchy endosperm of all varieties showed a common pattern of cell arrangement in which a central core of small cells was surrounded by larger cells radially oriented (Figures *2*, 3). Cells of the center were nearly isodiametric, ranging in size from about 45×50 to 80×105 microns. Length-width ratios of these cells in cross section were approximately 1.0-1.2, and the areas 2250 to 8400 sq microns. No relationship could be demonstrated between the measurements of central cells and grain length.

Cells immediately lateral to the central core were larger, and either isodiametric, slightly elongated, or slightly flattened radially (estimated ratio 0.7-1.4). Cells adjacent to the aleurone were smaller and many were flattened radially (estimated ratio 0.2-1.0) (Figure 4D, H).

Cells immediately dorsal and ventral to the core were various in size, especially in the radiating dimension (Table 1; Figures *2*, 3). Generally the radiating dimensions of these cells were shorter in long-grain varieties than in medium- and short-grain varieties; however, this relationship was only moderately strong. Ratios of radiating to sectoring dimensions likewise corresponded roughly to grain length. Cell areas were at variance with ratios, and showed no relation to grain length.

The value of cell dimensions in characterizing rice varieties could not be determined with the small number of replications investigated.

Endosperm cell walls, in sections treated to remove the contents, were stained by the ferric-fericyanide reagent (Figure 4H, I); whether this indicates the presence of amino acids, pectins,[c] or other substances has not been determined. The walls were also stained by ruthenium red (J), which suggests pectin or hemicellulose, and by the zinc-chloride-iodide reagent (K) which suggests cellulose. Appearance in polarized light (L, M) also indicates the presence of cellulose. The occurrence of a middle lamella and two primary wall layers was suggested in some preparations but not established as common. Although endosperm cell walls were very thin (about 0.25 microns), the

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[b] The ferric-ferricyanide reagent, recommended by Lillie *(5)* for certain amino acids, is similar to Rawlins and Takahashi's ferrocyanide reagent *(11)* for pectic substances.

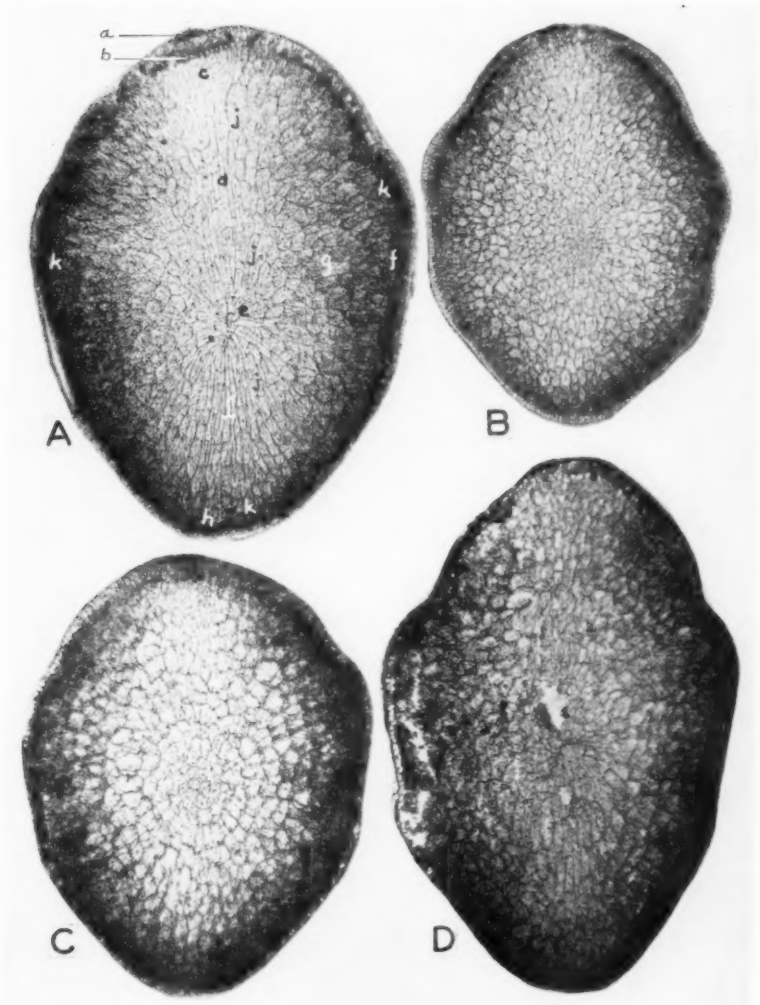


Figure 2. Freehand cross sections of brown rice after enzymatic removal of starch and staining with protein stains. A, C, and D were fixed in 10% formalin solution, B in modified Newcomer's fixative. A and B were stained with erythrosin B, C and D with erythrosin B and ferric ferricyanide reagent. A, Calrose (medium); B, Bluebonnet 50 (long); C, B455A1-25 (long); D, Early Prolific (medium). a, dorsal "ridge" pericarp; b, "ridge" aleurone; c, dorsal-peripheral (far-dorsal) area of starchy endosperm; d, dorsal area; e, central core; f, lateral-peripheral (far-lateral) area; g, lateral area; h, ventral-peripheral (far-ventral) area; i, ventral area; j, protein-poor tissue; k, protein-rich tissue. Magnification x 262.5.

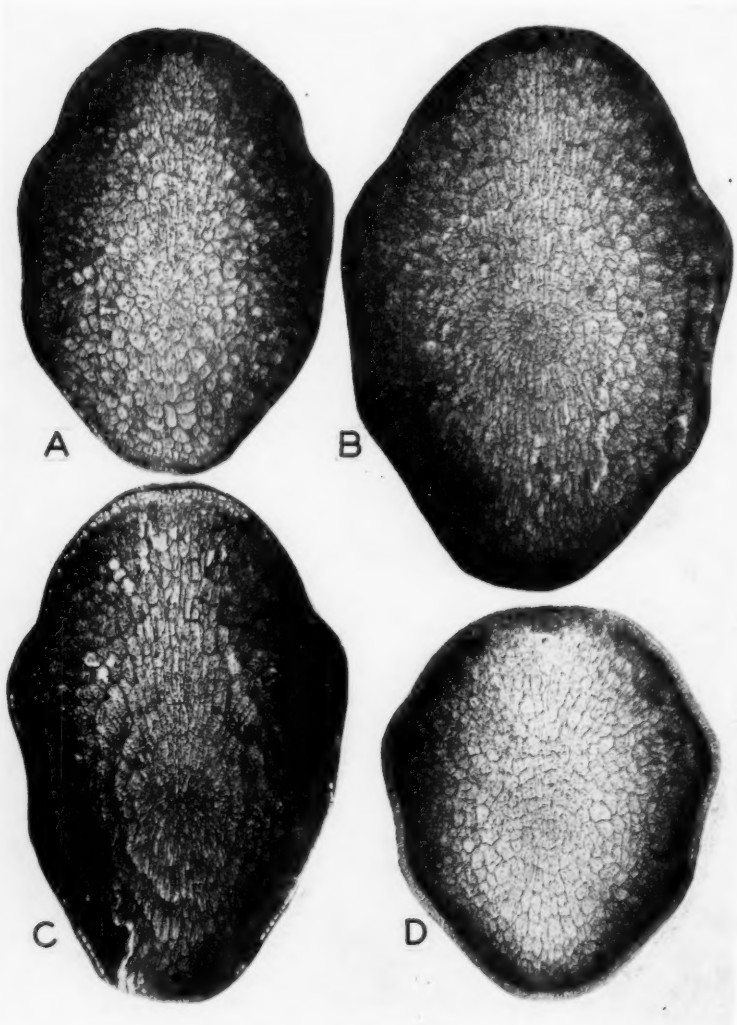


Figure 3. Freehand cross sections of brown rice varieties, after enzymatic removal of starch and staining with protein stains. A, B, and C were fixed in 10% formalin solution, and stained with erythrosin B and ferric ferricyanide; D was fixed with modified Newcomer's fixative and stained with erythrosin B. A, Fortuna (long); B, Caloro (short); C, Zenith (medium); D, Improved Bluebonnet (long). Magnification x 262.5.

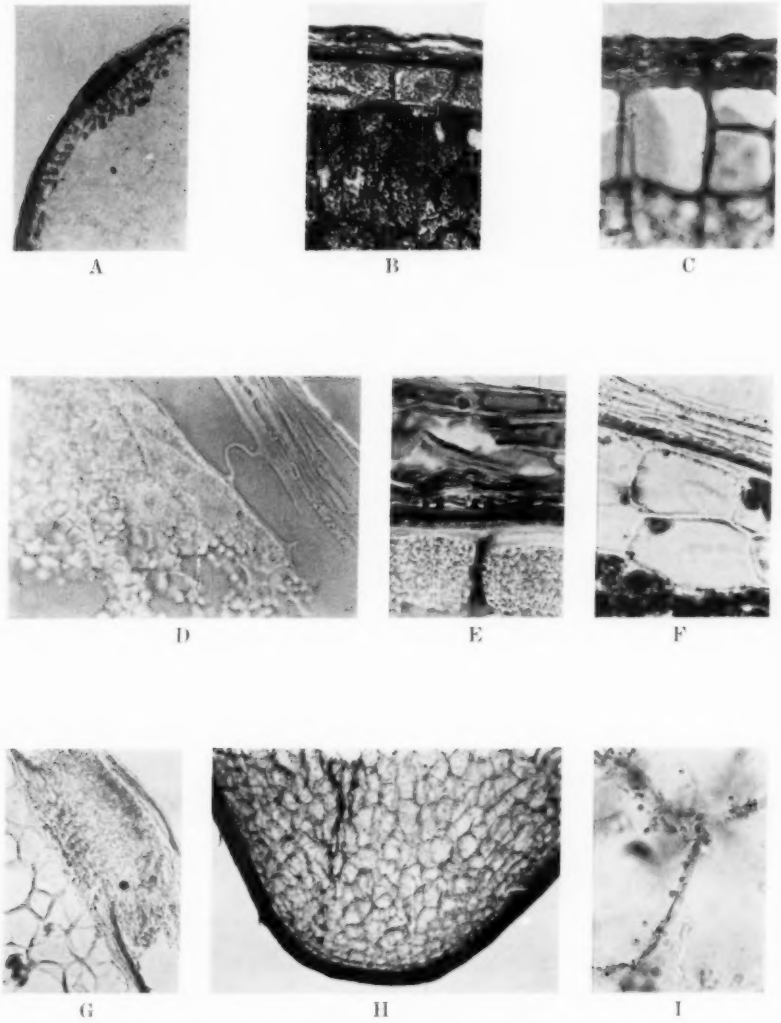
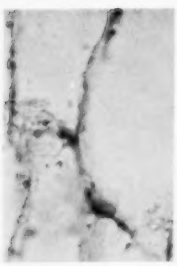


Figure 4. Color photomicrographs illustrating histochemical nature of rice kernels.

First Row: A, Bran layers (pericarp and aleurone) at the dorsal ridge. Calrose, raw, 10% glycerine; saliva, pepsin; oil red O. x 89.6. B, Bran layers (pericarp, testa, and aleurone). Blue Rose, cooked, Newcomer's fixative; oil red O, ferric-ferricyanide, iodine. x 387.2. C, Bran layers. Zenith, raw, Newcomer's; ruthenium red. x 972.

Second Row: D, Bran layers and cells from peripheral starchy endosperm. Zenith, raw, formalin; no stain; photographed with polarized, first order red illumination. x 972. E, Bran layers. Early Prolific, cooked, formalin; saliva; oil red O, zinc-chloride-iodide. x 972. F, Bran layers and cells from peripheral starchy endosperm. B4512A1-20, raw, 10% glycerine; oil red O, iodine. x 972.

Third Row: G. Dorsal ridge. Caloro, raw, Newcomer's; oil red O. x 972. H, Starchy endosperm, ventral area. B4512A1-20, raw, 10% glycerine; saliva, pepsin; oil red O, ferric-ferricyanide. x 89.6. I, Cell wall in starchy endosperm. B4512A1-20, raw, 10% glycerine; saliva, pepsin; oil red O, ferric-ferricyanide. x 972.



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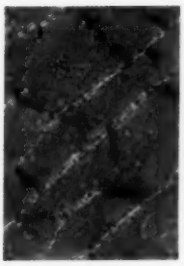

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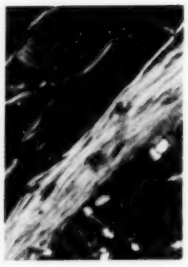

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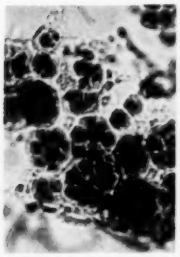

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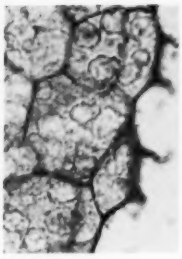

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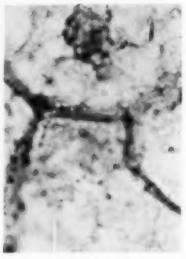

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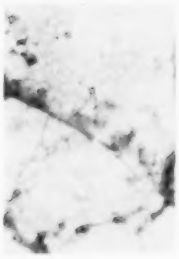

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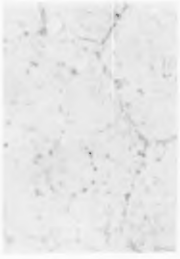

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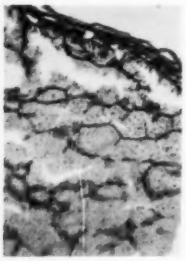

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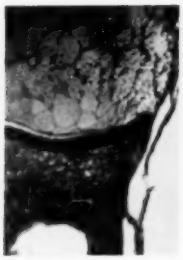

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impression was gained that they might be thicker or stronger in some areas of a cross section than in others, and possibly in some varieties than in others.

Diameters of individual starch granules ranged from 2 to 9 microns and averaged between 5 and 6 microns, regardless of variety. Starch granules were small, 2-4 microns in the peripheral cells of the starchy endosperm (D, F); they were fewest in lateral and ventral peripheral cells, forming tiny clusters well separated by the surrounding dense protein material. Starch granules of the major, central portion of the kernel were larger (5-9 microns and occurred in closely packed compound groups, several groups in each cell (N). Extremely small granules were found in cells near the germ, usually considered devoid of starch (O). Granule size may influence properties of the starch *(12)*.

Protein in noteworthy amounts was demonstrated in sections divested of starch and stained with erythrosin B or ferric-ferricyanide reagent.[c] Within a section, protein was most concentrated in peripheral-lateral and peripheral-ventral cells where starch granules were fewest, was least concentrated in peripheral-dorsal cells, and was often rather sparse in central, dorsal, and ventral cells (Figures 2, 3, 4 P). Within the cell, protein was most concentrated just within the wall; central bodies suggesting crushed nuclei (Q) were prominent in some varieties, obscure or absent in others. Each cluster of starch granules and apparently each individual granule was encased in a protein film (R); however, the individual films were difficult to demonstrate in protein-poor areas of some varieties.

Numerous fatty globules appeared in sections of rice softened in glycerine solution, divested of starch, and stained with oil red O (S). Their position, surrounding cavities created by removal of the starch, suggests that fats occur in combination with the proteins.

Structure of cooked rice. When rice was cooked, a certain amount of swelling occurred and the pressure of expanding starch caused breaks in the surface of the kernels. The extent and pattern of disruption depended on whether the rice was brown or milled, as well as on variety. In brown rice, a longitudinal fissure commonly occurred along the ventral side (Figure 5 A, B). Other forms of kernel disruption in brown rice included splitting of the bran at irregular points around the surface (C), detachment of the bran, and a tendency for the starchy endosperm to slough.

[c] This material could also be stained with ruthenium red.

Fourth Row: J, Cell wall in starchy endosperm. Zenith, raw, glycerine; saliva, pepsin; ruthenium red. x 972. K, Cell walls from starchy endosperm. Colusa, cooked, formalin; saliva, pepsin; zinc-chloride-iodide. x 972. Caloro, raw, 10% glycerine; saliva; zinc-chloride-iodide. x 387.2. L, Cell walls from starchy endosperm. Blue Rose, raw, 10% glycerine; saliva; zinc-chloride-iodide; photographed with polarized, first order red illumination. x 972. M, Cell walls from starchy endosperm (upper left and center diagonal) and scutellum (lower right). Colusa, cooked, formalin; saliva, pepsin; zinc-chloride-iodide; photographed with polarized illumination. x 972.

Fifth Row: N, Starch granules of average size. Calrose, raw, 10% glycerine; oil red O, iodine. x 972. O, Starchy endosperm at germ attachment region. Caloro, raw, Newcomer's; oil red O, iodine, x 972. P, Cells from lateral-peripheral area of starchy endosperm. Century Patna 231, raw, glycerine; saliva; ferric-ferricyanide. x 972. Q, Cells from starchy endosperm near the center. Century Patna 231, raw, glycerine; saliva; ferric-ferricyanide. x 972.

Sixth Row: R, Cells from starchy endosperm near the center. Sunbonnet, raw, Newcomer's; saliva; erythrosin B, ferric-ferricyanide; permanent slide. x 972. S, Cell from starchy endosperm near the center. Calrose, raw, glycerine; saliva; oil red O. x 972. T, Aleurone grains. Rexoro, cooked, Newcomer's; oil red O, ferric-cerricyanide. Nato, cooked, Newcomer's; oil red O, ferric-ferricyanide; photographed with polarized illumination. x 972. U, Starchy endosperm showing detachment of bran caused by cooking. Nato, cooked, Newcomer's; erythrosin B, ferric-ferricyanide, dilute iodine. x 387.2.

Seventh Row: V, Portion of starchy endosperm and germ. Century Patna 231, cooked, Newcomer's; erythrosin B, ferric-ferricyanide, iodine. x 89.6. W, Starchy endosperm at breach caused by cooking. Improved Bluebonnet, cooked, Newcomer's; oil red O, iodine solution. x 89.6.

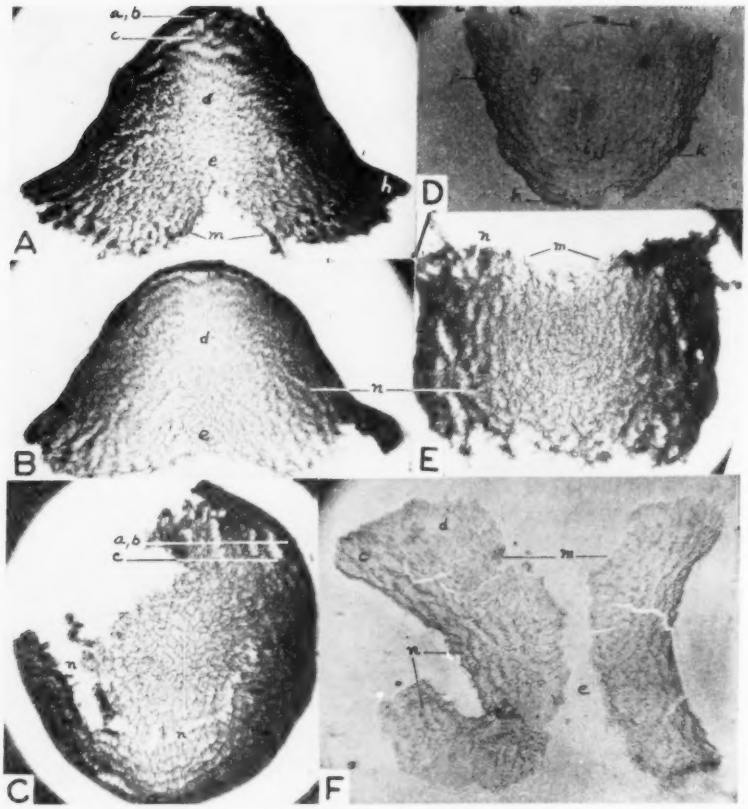

Figure 5. Cross sections of cooked brown and milled rice, variously treated. A, Rexoro (long), brown, cooked, Newcomer's, oil red O, ferric-ferricyanide; B, Improved Bluebonnet (long), brown, cooked, Newcomer's, erythrosin B, ferric-ferricyanide; C, Texas Patna (long), brown, cooked, Newcomer's, erythrosin B, ferric-ferricyanide; D, and F, milled, cooked, formalin, iodine vapor and acid fuchsin (permanent mounts); E, Texas Patna (long), milled cooked, fixed in modified Newcomer's solution, and stained with erythrosin B and ferric-ferricyanide. a, dorsal "ridge" pericarp; b, "ridge" aleurone; c, dorsal-peripheral (far-dorsal) area of starchy endospem; d, dorsal area; e, central core; f, lateral-peripheral (far-lateral) area; g, lateral area; h, ventral-peripheral (far-ventral) area; i, ventral area; j, protein-poor tissue; k, protein-rich tissue; m, radiating walls ruptured; n, sectoring walls ruptured. Magnification x 162.5.

Aleurone grains (Figure 4 A, F) were always birefringent in cooked rice (T), never in uncooked rice.

In milled rice, disruption was more complete than in brown rice after a given cooking time. Longitudinal fissures occurred always along the dorsal line, in contrast to the condition in brown rice, and also usually along the ventral line (Figure 5 D, E). The fissures were shallow, or extended from the dorsal side to the core, or from the ventral side to the core, or from the dorsal to the ventral side to result in splitting or halving of the kernels (F). In gross observations it was determined that simple longitudinal fissuring with a tendency to split was characteristic of most of the long-

grain varieties, while splitting accompanied by crosswise segmentation or sloughing occurred in most medium- and short-grain varieties.

Longitudinal fissures in the starchy endosperm involved the rupture of sectoring cell walls by the expanding starch in affected cells. Halving of the kernels may have been favored in some cases by the pre-formed internal rift. Detachment of the bran and sloughing of endosperm tissue involved rupture of radiating rather than sectoring cell walls (U). No evidence of cell separation along a middle lamella was observed. No regular longitudinal fissures, due to rupture of sectoring cell walls, were observed along the sides of cooked rice.

Apparently the gelatinizing starch tended to fill the space available to it. Cooked starch filled the cells adjacent to the germ (V) and showed a pale reaction to iodine. Cooked starch where exposed by rupture also showed a paler iodine reaction than that in the interior portion of the kernel (V, W). Dissolved amylose appeared as dark blue spots of irregular shape, contrasting with the usual lavender or purple color of intact cooked starch.

DISCUSSION

Obviously rice when cooked did not expand in proportion to the swelling capacity of its starch, which may be as much as 64 times in volume *(8)*. Suppression of swelling and the different patterns of disruption may be attributed largely to delaying or limiting effects of non-starch components, starch theoretically having greatest freedom to expand in situations where combined limiting components are weakest. The thick cell walls and corky layer of the bran delay water penetration, resist rupture, and restrict expansion of the starch within, especially along the thick dorsal ridge. In the starchy endosperm, cell walls and protein may limit the expansion of starch and also determine the position of cleavages in the tissue. The "barrier value" of cell walls may be related to thickness, composition, and distance apart; that of protein may be related to concentration and composition.

Within a section, conditions in the endosperm most favorable for starch expansion and tissue splitting are exemplified in the dorsal and ventral cells of such varieties as Calrose (Figure 2 A), where sectoring walls are far apart and protein sparse. The converse is observed in peripheral-lateral cells, where cell walls are closer together and protein more plentiful. The universal occurrence of a dorsal lengthwise fissure in the endosperm of cooked milled rice may be connected with low protein content and apparent weakness of cell walls along the dorsal line, conditions which seemingly counteract the small size of cells in that postion.

A relationship of non-starch components with disruption patterns was demonstrated for brown as opposed to milled rice, and for the several locations within a kernel. However, an appraisal of the cooking behavior of rice varieties as influenced by non-starch entities must await additional observation at both gross and microscopic levels. More detailed histochemical studies of the cell walls (thickness, area, and composition), the protein (composition and distribution), and the fat and mineral content, should help to clarify the cooking characteristics of different rice varieties.

SUMMARY

Cross sections from 23 varieties of uncooked and cooked brown and milled rice, after histochemical treatments and staining to demonstrate starch, protein, fats, and cell wall materials, showed anatomical characters which help to explain the appearance of the cooked kernels.

The pericarp and aleurone, two layers of the bran, were thickest along

the dorsal "ridge," and were separated by a third, moisture-resistant corky layer. In the starchy endosperm, cell sizes and shapes differed according to position within a section and according to variety. Starch granules were smaller and less crowded in peripheral cells than elsewhere. Protein material lined endosperm cell walls and encased all starch granules; it was most plentiful in cells where starch was least crowded, and was more prominent in some varieties than in others. Cell walls appeared fragile and were best observed in sections divested of both starch and protein. Their reactions to various stains indicated presence of cellulose and pectic substances.

Disruption of brown rice kernels during cooking was affected by the bran with its thick dorsal ridge and water-resistant corky layer, which delayed water penetration and limited the expansion of gelatinizing starch. Cell walls and protein of the starchy endosperm also may have had considerable value in restricting starch expansion and in regulating the pattern of kernel disruption.

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INFLUENCE OF FREEZING AND THAWING ON HYDRATION AND CHARGES OF THE MUSCLE PROTEINS[a]

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The relation between the changes of hydration and the changes in charge of muscle during the heating of meat has been reported previously *(13)*. This investigation showed that it is possible to study the protein denaturation of meat by means of the determination of its water-holding capacity and its buffer capacity at different pH values and of the amount of dyes bound by the acidic and basic groups of the proteins. In the present paper these methods are used to determine whether freezing and thawing cause denaturation. This problem was of interest because of the undesirable changes in meat proteins brought about by freeze drying *(10)*. In this process the meat is frozen before drying and the question arises whether changes caused by freeze drying are due to the freezing procedure itself or to other factors.

It is known that freezing immediately after slaughter presents special problems because of the presence of adenosinetriphosphate (ATP) and the accelerated decomposition of the ATP during thawing resulting in "thaw rigor." In order to prevent such influences of ATP, for all experiments meat was used 5 to 6 days post mortem, after resolution of rigor mortis.

METHODS

The animals used were 5- to 6-year-old utility grade cows. Two to 3 lb of the *longissimus dorsi* muscle were cut out from the carcass 5 to 6 days after slaughter. Connective tissue and fat were removed as much as possible. Some samples of muscle were ground twice in a cooled meat grinder and minced for 30 sec in a cooled Waring blender. The temperature immediately after mincing was about 20° C. Other samples of muscle were cut into 1.0-1.5 cm cubes.

Quick-freezing of meat. Fifty g of ground or cut meat was frozen in thimble-shaped tubes, 3.5 × 16.0 cm, by placing the stoppered tubes in an acetone-CO_2-mixture at about −55° C for 15 min. The frozen meat was thawed by putting the tubes in water at room temperature for 45 min. With respect to meat hydration, thawing at room temperature is preferred to thawing at lower temperatures *(1, 2)*. The ground, thawed meat was mixed thoroughly. The cut, thawed meat was ground in the same manner as the fresh meat.

Slow-freezing of meat. Meat cut in cubes was placed in glass stoppered bottles and kept at −15° C. The meat was frozen in about 2 hr. After 4 hr the muscle was thawed by putting the flasks in water at room temperature for 45 min. This meat was ground in the same manner as the fresh meat. Before grinding, the small amount of "drip" that had separated was added back to the meat after the first grinding in the meat grinder. The temperature after grinding was never higher than 20° C. The moisture was determined in the fresh ground meat and in the thawed ground meat.

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TABLE 1

The influence of different methods of freezing on the water-holding capacity and the pH of meat

Meat No.	State of meat during freezing	Temperature of freezing ° C	Moisture		pH		Water-holding capacity			
							Press method no added water		Centrifuge method, 70°	
							Bound water, % of meat		Bound water, % of meat	
			Fresh meat %	Thawed meat %	Fresh meat	Thawed meat	Fresh	Thawed	Fresh	Thawed
A	Ground	−55	75.21	75.21	5.59	5.54	49.7	53.0		
X	Ground	−55	75.13	75.09	5.53	5.46	57.7	58.3	44.7	45.9
XI	Cubes	−55	75.31	75.49	5.71	6.18	53.0	60.3	55.7	55.9
XII	Ground	−55	71.86	72.20	5.47	5.42	61.0	63.0	48.9	50.9
XII	Cubes	−55	71.86	71.71	5.47	5.43	61.0	62.4	48.9	48.9
XII	Cubes	−15	71.86	72.01	5.47	5.42	61.0	59.7	48.9	47.0
XIII	Cubes	−55	74.50	74.30	5.52	5.62	53.7	54.4	43.9	43.9
XIII	Cubes	−15	74.50	74.82	5.52	5.53	53.7	49.7	43.9	42.0
XIV	Cubes	−55	74.35	74.60	5.44	5.45	52.3	53.4	42.4	42.8

Analysis of fresh, frozen, and thawed meat. All methods have been described previously:

1. Determination of moisture *(13)*.
2. Water-holding capacity, determined by pressing meat on filter paper at room temperature *(9)*.
3. Water-holding capacity, determined by centrifuging of meat ("tube method") after heating at 70° C *(24)*. In this case, "bound water" is the water retained by the tissues after heating. The water-holding capacity is expressed as bound water, related to 100 g of the fresh muscle.
4. Influence of pH on the water-holding capacity of meat containing 60% added water. Determination of the isolectric point (I.P.). (Press method). *(11)*.
5. Buffer capacity of muscle tissue at different pH values *(12)*.
6. Determination of acidic and basic groups in muscle by binding of dyes *(13)*.
7. Determination of protein in muscle *(13)*.
8. Determination of pH values in muscle and in the juice of the "tube method" *(13)*.

RESULTS AND DISCUSSION

Influence of temperature of freezing and shape of meat on the water-holding capacity and pH values of frozen and thawed meat. Freezing and thawing caused only minor changes in the water-holding capacity of meat (Table 1). Quick-freezing (−55° C) of both ground and cut meat results in a small but significant increase of water-holding capacity. This confirms an earlier report from this laboratory *(24)*. There is no significant influence of the shape of meat (ground or cut in cubes) on the hydration of the thawed tissue.

By contrast, slow freezing of meat (−15° C) decreases the water-holding capacity slightly. Similar results were obtained when increasing amounts of water were added to the samples. Table 2 shows the result of one of two series.

It is known that slow freezing results in more "drip" than quick freezing *(3, 4, 6, 14, 22)*. The more slowly the tissue is frozen the larger are the crystals of ice and more cell walls are destroyed *(16)* and large crystals of ice are found between the muscle fibers. In fast freezing, however, water inside the cells is frozen in small crystals *(17)*. But it is surprising that the

TABLE 2

The influence of freezing-temperature and shape of meat on the water-holding capacity (press method) of thawed meat (meat No. XII)

Shape of meat during the freezing	Temperature of freezing C°	Added water %	pH	Bound water, related to meat %
Ground		0	5.52	53.7
Ground		20	5.52	56.4
Ground		40	5.53	53.3
Ground		60	5.54	53.5
Cubes	−55	0	5.62	54.4
Cubes	−55	20	5.63	58.5
Cubes	−55	40	5.61	55.1
Cubes	−55	60	5.62	52.5
Cubes	−15	0	5.53	49.7
Cubes	−15	20	5.52	51.6
Cubes	−15	40	5.51	49.1
Cubes	−15	60	5.52	44.9

water-holding capacity of some meats is increased by freezing and thawing. Perhaps the formation of tiny ice crystals without a destruction of cell walls results in a loosening of the protein structure and, therefore, a certain increase in charged, water binding groups on the proteins. Fischer and Jensen *(8)* found that a "destruction" or loosening of protein structure by freezing increases the amount of water bound by the tissue after thawing. Hiner *et al (14)* believe that a "rupturing" of fibers occurs by intracellular freezing permitting the proteins to reabsorb a large proportion of the water originally frozen in the meat.

There was no significant influence of freezing and thawing on the pH values of meat. In some cases there was a slight increase of pH and in other cases a slight decrease. There was also no significant influence of freezing on the pH-shift in the meat by heating at 70° C.

Influence of pH on the water-holding capacity of fresh and of quick frozen and thawed meat. Three series of experiments with one ground and two cut meats were made to investigate the influence of freezing and thawing on the isoelectric point (I.P.) (pH of hydration minimum) and the protein charges available for binding water. Figure 1 shows the results of one of

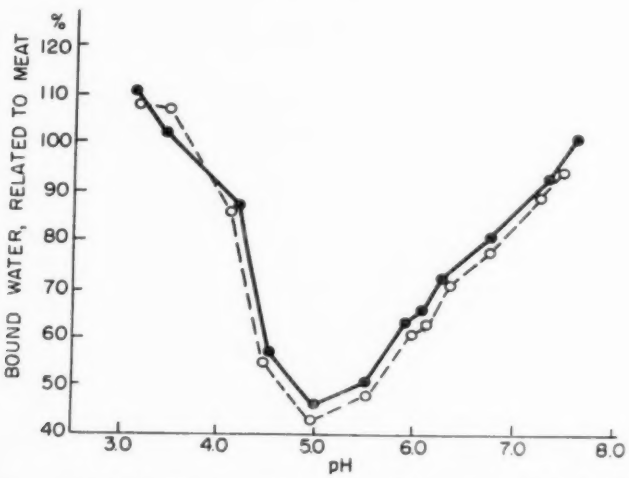

Figure 1. The relation between pH and water holding capacity of fresh (●) meat and the same meat after freezing and thawing (○).

these experiments. There is no significant influence of quick-freezing and thawing on meat hydration either in the isoelectric range nor in the basic or acidic range of I.P. According to these results, quick-freezing does not cause any shifts of I.P. or any marked changes of available protein charges. A small decrease of water-holding capacity by freezing was observed in one experiment, but in other experiments no decrease of hydration occurred.

Influence of pH on the buffer power of fresh, quick-frozen and thawed

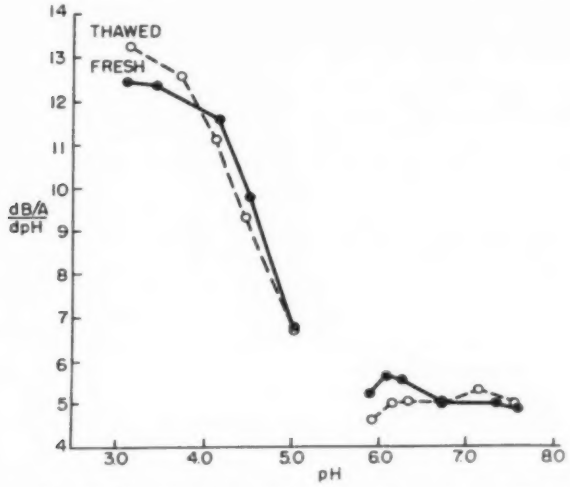

Figure 2. Relation between quick freezing and the buffer capacity of (●) fresh and (○) quick frozen and thawed meat.

meat. Two experiments with cut and one with ground frozen meat showed no significant influence of quick-freezing on the buffer capacity of tissue. Figure 2 shows the results of one of these experiments. The small increase of buffer power at pH<4 is not significant. Because changes of buffer power in the whole tissue are insignificant, it is to be expected that there would be no changes in the buffer capacity of water-soluble proteins and non-proteins. Therefore, water extracts and trichloracetic acid extracts of tissue were not studied. These results agree with those obtained by the determination of water-holding capacity at different pH values. There is no significant change of protein charges by freezing and thawing.

Influence of quick-freezing and thawing on the number of acidic and basic groups in muscle proteins. As Table 3 shows, quick-freezing and thawing of meat produces no great change of acidic or basic groups in the muscle tissue. Nevertheless, the small increase of charged groups caused by freezing and thawing appears to be significant. These results agree with the findings obtained by measurements of meat hydration where it was established that the "loosening" of protein structure produced by quick-freezing causes a small increase of free protein charges available for binding water.

TABLE 3

Influence of quick-freezing and thawing on the amount of acidic and basic groups of the muscle proteins [1]

Treatment	Equivalents acidic groups per 10^4 g protein	Equivalents basic groups per 10^4 g protein
Fresh	15.5 (0.8)	13.1 (0.2)
Thawed	15.8 (0.6)	13.3 (0.2)

[1] The figures in parentheses are the standard deviations.

It was shown *(13)* that mild heating of meat (30°–40° C) results in characteristic changes of hydration caused by protein denaturation. At temperatures >40° C protein charges and, therefore, pH and I.P. of muscle are changed. Quick-freezing and thawing, however, cause no significant changes either of hydration or of I.P. and pH. Changes of acidic and basic groups are very small and relatively insignificant. Therefore, quick-freezing and thawing of meat do not result in any considerable denaturation of muscle proteins.

The determination of the ATP-ase activity, the solubility, and the swelling of tissue in salt solutions *(5, 6, 7, 18, 21, 22, 23)* also showed that freezing at low temperatures (−25 to −60° C) results in no denaturation of muscle proteins. Seagran *(19)* reported that freezing itself produces some subtle changes in the structure of actomyosin present in post rigor fish muscle yielding a more symmetrical molecule. Dubuisson *(7)*, in his electrophoretic studies, recommends freezing slices of tissue and then extracting because this would be the most gentle method. Callow *(4)* points out that during the formation of ice in meat the salts soluted in the muscle juice become more concentrated. At high concentration, salts may be capable of denaturing some cellular proteins *(16)*. According to our experience, the interaction between salt ions and muscle proteins occurs rapidly. The normal process of shrinking or coagulation of muscle proteins by high salt concentration cannot occur during quick-freezing because after a short time of storage in frozen state (2 hr) no change of hydration occurs. Probably the rate of the formation of ice crystals does not permit a high concentration of salts to develop.

Storage of meat for long periods of time may cause changes of meat proteins *(20, 23)*, for example, by the effect of some enzymes which still retain some activity at low temperatures *(15)*. However, freezing and thawing do not in themselves cause excessive denaturation of meat proteins.

CONCLUSIONS

Quick-freezing and thawing of beef result in no considerable denaturation of muscle protein.

Quick-freezing causes a very small but significant increase of the water-holding capacity of meat, probably by a mechanical loosening of tissue-structure due to the formation of tiny ice crystals inside the cells.

Slow-freezing causes a significantly small decrease of the water-holding capacity of meat, probably due to some destruction of protein structure by formation of large ice crystals between the cells.

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RHEOLOGY OF COCOA BUTTER. II. EFFECT OF STORAGE TEMPERATURE ON APPARENT VISCOSITY

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In a previous study *(12)*, it was shown that molten cocoa butter, on standing at temperatures near the solidification point for periods up to 13-14 hours, became more viscous, even before the appearance of microscopically visible crystals. This phenomenon was interpreted as the consequence of submicroscopic molecular aggregation. Presumably the major portion of this aggregation was of an orderly nature, such as might precede the organization of crystalline nuclei.

As the samples were kept for long periods of time, some yellowing and some increase in viscosity began to occur. It is well-known that purified fats, which are composed of unsaturated fatty acids, may be oxidized during storage. The possibility of oxidation in cocoa butter resides in the presence of large amounts of oleic acid in its component fats. According to most recent evidence *(8)*, the major constituents of cocoa butter are the di-saturated triglycerides, 2-oleopalmitostearin (55%) and 2-oleodistearin (20%). The presence of small amounts of linoleic *(1, 4)* and possibly even linolenic *(3)* acid radicals has also been reported. Nevertheless, Bailey *(1)* states that cocoa butter ordinarily is rather resistant to oxidation.

Because of the contradictory nature of this evidence, further study of the oxidative relationship seems to be indicated. Oxidation frequently results in a condensation or polymerization of the unsaturated moieties *(1, 10)*. If this does occur, the viscous properties of a fat, which undergoes oxidation-induced polymerization, should change markedly. Since oxidative processes have a pronounced temperature dependence *(1, 7)*, this study has been undertaken to explore the roles of temperature and time in the viscous properties of cocoa butter.

MATERIALS AND METHODS

A fresh sample of cocoa butter was prepared by solution, clarification with activated charcoal, filtration, and solvent evaporation, in several replications *(12)*. Then the still molten fat was centrifuged, and the centrifuge tubes were stored in a vacuum desiccator at 0° C. In contrast to the starting preparation, the purified cocoa butter was colorless in the molten state and white when crystallized. (The original fat was quite yellow under both conditions.) Moreover, whereas the original product had a definite chocolate odor, the purified fat was odorless.

At the start of the experiment, the stored cocoa butter was melted at 60° C, and 5 ml were pipetted from the top of a centrifuge tube into the reservoir of each of 6 viscometers. Preliminary control readings were made at 31° C. The viscometers were then stored at 0, 17.5, 30, 45, and 60° C. At each storage interval (1, 2, 3, 5, 10, 30, 60, and 90 days), the samples were melted if necessary (at 60° C for up to an hour) and equilibrated at 31° C for an hour. Viscosity readings were then made. Between successive readings, the viscometers and their contained samples were stored at their respective holding temperatures. The 60° C sample was held further until 270 days for a final testing.

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The viscometers which were used for the experiments were Ostwald-type pipettes (BS design). The outflow time of water in these ranged from 76.1 to 88.0 seconds at 31° C. Because the viscosity of cocoa butter is about 90 times that of water at this temperature, the viscometers were connected to a pressure system *(12)*. A pressure regulator, which was inserted between the compressed air source and the pressure reservoir, maintained a pressure of 20.10 cm of Hg within ±0.01 cm. Only the emptying time was used for the outflow of the fat.

The control outflow times at the beginning of the experiment were taken to represent an arbitrary apparent viscosity of 1.00. Flow readings at different storage periods for different storage temperatures were then converted to apparent relative viscosities: $\eta_{rel} = \frac{t_x}{t_o}$, where t_o is the initial (control) outflow time and t_x is the time of outflow at any given storage condition x. Inasmuch as cocoa butter has been shown to behave as a non-Newtonian fluid *(12)*, a "one-point" reading cannot serve to define its complete rheological behavior. However, since the shear stress here is always held constant, the determination of shear rate has a valid comparative significance. In addition, at 31° C the shear rate-shear stress relationship is virtually rectilinear *(12)*, and it intersects the origin of a graph of these variables.

RESULTS AND DISCUSSION

In all cases, the relative apparent viscosity increased throughout the period of storage (Table 1). The least change occurred at 0° C and the greatest

TABLE 1

Relative apparent viscosity of cocoa butter

Time of storage (days)	Temperature of storage (C °)				
	0	17.5	30	45	60
0	1.00	1.00	1.00	1.00	1.00
1	1.04	1.02	1.02	1.04	1.05
2	1.06	1.04	1.04	1.06	1.09
3	1.07	1.06	1.05	1.08	1.11
5	1.07	1.07	1.06	1.09	1.13
10	1.09	1.10	1.09	1.14	1.22
20	1.09	1.13	1.10	1.19	1.35
30	1.10	1.17	1.13	1.26	1.49
60	1.10	1.23	1.19	1.43	1.82
90	1.10	1.27	1.28	1.60	2.18
270	....	....	....	....	5.88

change at 60° C. At intermediate temperatures, the amount of change fell between these two extremes. Thus, both the temperature of storage and the time of storage were directly related with the increase in viscosity. (Note that at 60° C, the viscosity was increasing at a faster rate in the period after 90 days than earlier).

Along with the changes in viscosity, two additional phenomena were positively related with storage temperature and time: (1) increase in intensity of yellowing; and (2) increase in degree of rancidification, as detected by odor and flavor. Furthermore, when the 60° C specimen of 270 days was cooled to 0° C, it displayed many gel-like properties; and, unlike the other storage specimens, it was plastic rather than brittle at 0° C.

The increasing yellowness and rancidity of the cocoa butter, as a function

of time and temperature of storage, indicate clearly that oxidation is occurring. [Purely thermal polymerization *(11)* with a Q_{10} of about 2 *(9)* would be extremely minimal, even at the highest temperature and longest time used here.] Kleinert *(5)* has also demonstrated that a high temperature helps induce oxidative changes in purified cocoa butter (but not in unpurified samples!). Another result of oxidation is the increasing rate of change of viscosity at 60° C. This apparently substantiates the documentation *(1, 6, 10)* that fat oxidation is an autocatalytic process. Nevertheless, it is possible that some persistent natural antioxidants might be used up, or destroyed after an initial induction period at 60° C, with concomitant increase in rate of oxidative polymerization.

An increase in viscosity is an indication of an increase in particle size or asymmetry. The direct relationship with temperature seems to be due to polymerization of the (unsaturated) fatty acid radicals in the cocoa butter. [Because of the temperature of observation and the pronounced supercooling properties of cocoa butter *(2)*, it could not be expected that crystalline nuclei would develop during the period of time consumed in making the outflow measurements *(cf. 12)*, nor were such nuclei found.]

Increasing polymerization would account also for the gel-like or plastic properties of the 60° C sample which was held for 270 days. Thus, oxidized cocoa butter has been used as a crystallization inhibitor for other fats *(2)*. Natural anti-oxidants probably are present in chocolate *(cf. 1, 5)* and perhaps serve to prevent oxidative changes. The use of anti-oxidants should be an important consideration for products which contain cocoa butter and must be stored at high temperatures for prolonged periods.

The temperature dependence of the viscous changes also may be used to explain the small deviation of the 0° and 17.5° C samples from the general trend during the first part of the experiment. These samples always solidified during their storage and were therefore heated to 60° C for some time for complete melting before a viscosity measurement. They received far more such heating than did the 30° C sample. In fact, this latter remained wholly fluid up to 5 days at the start of the experiment; and even after 20 days, most of the sample in the viscometer was still fluid. A replicate of the 0° C sample, which was not removed from storage until being tested at 90 days, had a relative apparent viscosity of 1.07 (after being heated to 60° C for 90 min).

The results of this study also show that the contribution of polymerization to viscosity during the time period of the previous study *(12)* is negligible. Hence they serve to confirm the conclusion of that study, that at 24°-30° C an increase in viscosity over a short period of time is due to the organization of submicroscopic aggregations of fat molecules—presumably crystalline nuclei.

SUMMARY

Purified cocoa butter was stored at a series of different temperatures for periods up to 270 days. A constant increase in viscosity, yellowness, and rancidity was a direct function of both temperature and time of storage. Results were related to oxidative changes which brought about polymerization of the component fats.

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SETTING TIME AND SETTING TEMPERATURE OF PECTIN JELLIES

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Setting time is defined by Kertesz *(15)* as "the interval of time which elapses between the instant at which all constituents of a jelly batch (sugar, pectin and acid) are placed in the final container after cooking and that instant at which jellification of the whole into a coherent mass may be observed."

However, it should be remembered that in some cases setting may occur during the cooking, as has been shown by Cole, Cox and Joseph *(4)*. For this reason it seems better to define setting time as the time between the moment that all ingredients necessary for forming the jelly are present in heated solution in the correct proportion and the moment that jellification into a coherent mass occurs.

Hinton *(13)* has stressed that the setting *temperature* of a jelly is shown to be a determinate physical property, whereas the setting *time* is a secondary property dependent on temperature conditions. According to Hinton the time of setting for a certain jelly batch is governed by the velocity at which the setting temperature of the mixture is reached. The more quickly a jelly batch is cooled, the lower the setting temperature may become due to undercooling. After determining the setting temperature of a batch at different cooling rates, it is possible to calculate by extrapolation the setting temperature when the cooling rate is zero. Hinton states that the setting temperature, calculated in this way and corresponding with infinitely slow cooling, coincides within experimental limits of accuracy with the re-melting temperature during heating.

Kertesz *(15)*, writing in 1951, noted a lack of published data on the setting time of pectin jellies. Methods for its determination had been described by Joseph and Baier *(14)* in 1949 and earlier, in 1939, by Olsen, Stuewer, Fehlberg and Beach *(18)* but data had not yet accumulated and this is relatively true today.

Olsen, Stuewer, Fehlberg, and Beach *(18)* have also described the influence of the degree of esterification of pectins, indicating that setting time is increased by lowering the degree of esterification from about 75% to about 60% and that further saponification tends to decrease the setting time. These studies were made with jellies at pH 3.1.

It is well known that the setting time of a jelly batch is influenced by the pH, a lower pH effecting a more rapid jellification *(7, 14, 15)*. Increasing the sugar content of the batch also has an influence, causing a decrease in setting time *(4)*.

According to Baker and Goodwin *(1)* high jelly grade pectins tend to show longer setting times. They found a relation also between degree of esterification and setting time, but stated that high-ester pectins are not always rapid-set pectins. Lampitt and Money *(16)* observed an increase in

the firmness of pectin jellies during 7-10 weeks of storage at 25° C and 30° C.

In the experiments being reported we studied setting times as well as setting temperatures of jellies with varying pH, made from pectins with different jelly grades or with different degrees of esterification. In some cases the influence of ash constituents and pectin concentration on setting temperature has also been studied.

METHODS

Preparation of jellies. It would be desirable to study the setting time or temperatures in pectin jellies with the same percentage of pectin and the same final firmness. However, this is impossible when comparing various types of pectins with differing degrees of esterification and polymerization, which influence the jellying power of the pectins and the relation between jellying power and pH and ash constituents.

The content of different pectins in the jellies corresponded to the amount necessary to give a gel of standard strength at a pH 2.0–2.4 according to the Wageningen-method for determination of jelly grade *(7)*. This method is based on the same principles used in the Cox-Higby sag method *(5, 14)*. The pectin is cooked with the sugar and immediately after cooking acidified to the desired pH with tartaric acid; the sugar content of the jellies is 65%. The influence of pectin content was studied by comparing, in some cases, the setting temperature of these jellies with jellies having a pectin content corresponding with the grades obtained by the modified Cox and Higby method, which has been used by Christensen *(3)*. It has been shown that a constant relationship exists between the results of both methods *(8)*, the commercial grades by Wageningen-methods being 1.58 times the modified Cox and Higby grades. This difference of jellying power between both grade values represents, generally speaking, the difference between European and American grades.

Determination of quantity, degree of esterification and jelly grade of pectins. The amount of "pure" pectins and their degree of esterification has been determined according to our modified titration method *(8)*. For determining jelly grades of the pectin preparations the Wageningen-method *(7)* has been used.

From these determinations the Wageningen-grades of the "pure" pectins, referred to in this publication, have been calculated.

Preparation of pectins with the same degree of esterification and a different jelly grade. For the study of the influence of jelly grade on setting time it is necessary to compare the behavior of pectins with the same degree of esterification *(15, 18)*.

It is possible to prepare pectins of various jelly grades (ranging from 180 to 580) without affecting their degree of esterification by the action of a commercial enzyme preparation (Filtragol) at 35° C at pH 3 *(8)*. Another possibility for partial decomposition of pectins, without influencing their degree of esterification, is by heating slightly acidified pectin solutions.

In Figure 1 the analytical data are shown for pectins which have been prepared by boiling aliquots of a 1, 15% pectin solution (degree of esterification 75%) for 15 min at pH values ranging from 3.0 to 7.5. The solution was buffered by 0, 37% lactic acid, citric and phosphoric acid, and before heating, the pH of each aliquot was adjusted to the different pH values mentioned above by using 20% NH_4OH. Nevertheless, the pH changed in some cases during boiling to a lower value, probably caused by a partial saponification of the pectin at the higher pH values.

It is obvious that at higher pH values a decomposition of the pectin occurred, which is indicated by diminishing jelly grade at a pH higher than about 4, and a measurable decrease of pectin content at pH higher than about 5.5. In this way, it is possible to prepare pectins with different jelly grade without any change of degree of esterification by boiling at a pH 4.0–5.5.

For our experiments pectins have been prepared with different degrees of polymerization by heating, by enzymic action, and by the action of ascorbic acid and hydrogen peroxide.

After this treatment the different pectins, made by methods listed above, were purified by precipitation in 70% acetone acidified with HCl to pH 1,5. The precipitates were pressed out and again dissolved. After a second precipitation and washing of the precipitate in pure acetone, an approximately 5 grade pectin solution was made and adjusted to pH 3.5 with 20% aqueous NH_4OH.

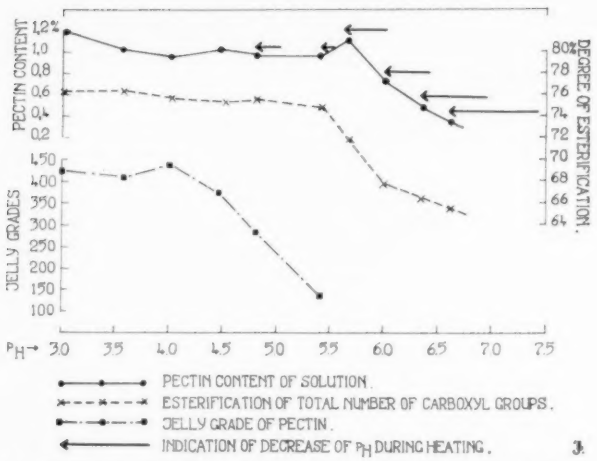

Figure 1. Influence of boiling during 15 minutes at different pH-values on the jelly grade, degree of esterification and amount of a pectin.

Preparation of pectins with different degrees of esterification. Pectins with different degrees of esterification have been prepared from an apple pectin solution (degree of esterification 75%, jelly grade 453) by saponification for different times at pH 1.0 and 60° C. After various intervals of time, the pectin in aliquots of the solution was precipitated with acetone and purified as described above, to give pectins with different jelly grade. It has been reported *(7)* that during such a saponification in acid environment, no appreciable decomposition of the pectin chains occurs.

In the trials concerning setting time and setting temperature, the pectins were used in the jellies at levels corresponding to their grade at pH 2.0–2.4. Although there is no change in chain length, the jellying power within this pH range is influenced by alteration of the degree of esterification.

The relation between jellying power and pH of pectins is also influenced by their degree of esterification, that is lower degrees of esterification give weaker jellies in the high pH region.

It was mentioned in the introduction that it is impossible to maintain a constant amount of pectin and a constant firmness of the jellies, when comparing the setting temperatures or setting times of jellies with varying pH and using pectins with different degrees of esterification.

Figure *2* shows the relation between jellying power and pH of jellies made from pectins which we prepared by the saponification method as described above. The same relation is shown for some pectins even after addition of 0.002 M $CaCl_2$ to the jellies before cooking.

The jellying power of "pure" pectins is expressed in commercial grades according to the Wageningen-method. The jelly pH was adjusted with tartaric acid and the jellies were tested after 48 hr storage at room temperature.

Figure *2* shows that the jelly grade of pectins was increased by saponification. Further, it demonstrates the well known difference in the relation between gel strength and pH for pectins with different degrees of esterification.

The percentage of pectin in these jellies is given by the relation, 65/jelly grade at pH 2.0–2.5 = % pectin in the jelly.

Determination of setting temperature or setting time. Immediately after cooking and the addition of tartaric acid, the jelly batch was poured into 12 test tubes which were then closed with rubber stoppers. The weight of jelly in each tube was about 14 g. The manner of cooling was different in the various experiments and is described along with the results of the separate trials.

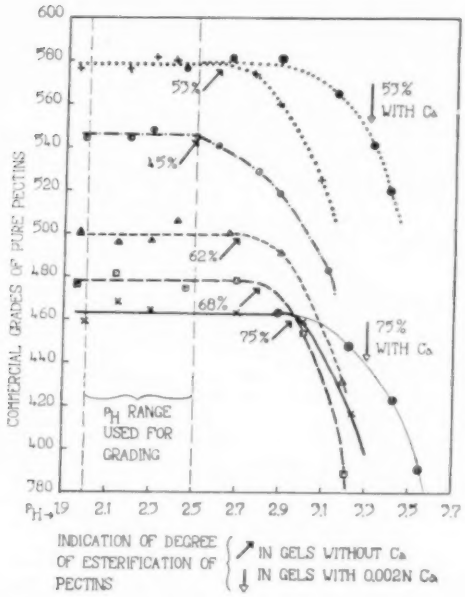

Figure 2. Relation between jellying power of pectins with different degree of esterification and the pH of the jellies.

After suitable intervals of time one of the tubes was turned upside down to see if a coherent mass in the tube had been formed. The tubes which had been used in this way were rejected for further use.

Setting time was considered as the time elapsed between the moment of addition of the acid after cooking and the moment that jellying to a coherent mass was observed. The temperature in the tubes at the moment of jellying, measured with thermocouples, was designated the setting temperature.

RESULTS

Setting temperatures of jellies made from pectins with different degrees of esterification. According to Hinton *(13)* pectin jellies are subject to undercooling under certain conditions. Therefore, the setting temperature of jellies having different pH was investigated. The results are shown in Figure 3, where setting temperature of jellies made from pectins with a degree of esterification between 52% and 68% is plotted against rate of cooling.

Hinton indicated that the points at which temperature curves cut the temperature axis (i.e. the setting temperature with infinitely slow cooling) should coincide with the re-melting temperature of the jellies.

It may be concluded from Figure 3 that the extent of undercooling at a certain cooling rate is increased by a higher pH of the jellies. This may be in agreement with the fact that Hinton observed a still more pronounced undercooling at pH 3.22. However, we have not been able to re-melt the jellies by heating to 100° C.

Since it is very laborious to calculate setting temperatures in large series of jellies by extrapolating to infinitely slow cooling, we used the slow cooling rate of about 0.4° C/min where undercooling was about a minimum.

We determined the setting temperatures of jellies from four pectins with different

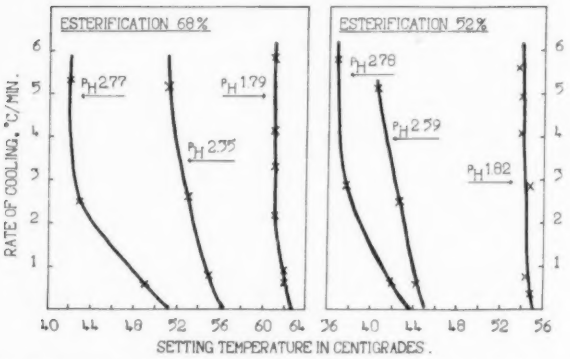

Figure 3. Influence of the rate of cooling in a waterbath on setting temperatures of jellies with different pH, made from two pectins.

degrees of esterification at different levels with and without the addition of 0,002 M $CaCl_2$ and in some cases with the addition of apple juice (the added amount of sugar being corrected for the sugar content of the apple juice). In two series, a higher percentage of pectin (1.58 × the amount indicated by the Wageningen-method of

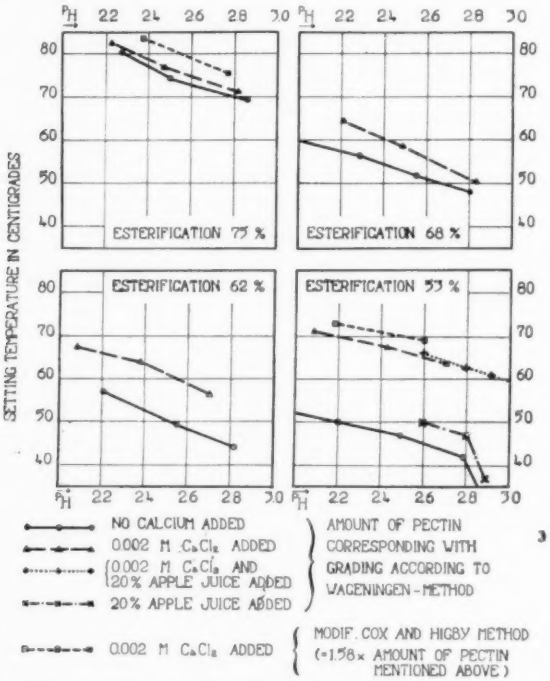
SETTING TEMPERATURE IN CENTIGRADES
ESTERIFICATION 75 %
ESTERIFICATION 68 %
ESTERIFICATION 62 %
ESTERIFICATION 53 %
NO CALCIUM ADDED
0.002 M CaCl2 ADDED
0.002 M CaCl2 AND 20% APPLE JUICE ADDED
20% APPLE JUICE ADDED
AMOUNT OF PECTIN CORRESPONDING WITH GRADING ACCORDING TO WAGENINGEN-METHOD
0.002 M CaCl2 ADDED
MODIF. COX AND HIGBY METHOD (=1.58× AMOUNT OF PECTIN MENTIONED ABOVE)

Figure 4. Relation between setting temperature and pH for jellies from pectins with different degree of esterification during cooling in a waterbath at 0.4° C/min.

grading) was used, which corresponded to the grade by the modified Cox and Higby method *(3)*.

The results, shown in Figure 4, indicate that within a wide range of pH a linear relation exists between setting temperature and pH as well for the gels with and without addition of calcium or apple juice. The slope of the curves, indicating this relation, is nearly the same for all pectins. The influence of the addition of calcium on setting temperature is dependent on the degree of esterification of the pectins. It is important to note that the setting temperaure of the jellies from the pectin having the lowest degree of esterification (53%) is raised considerably by the addition of calcium and under these circumstances is approaching the setting temperatures of a rapid-set pectin (75% esterification). From the results with pectin having an esterification of 53%, it is remarkable that here is no significant difference between the addition of 0.002 M $CaCl_2$ or the addition of the natural buffer of apple juice and 0.002 M $CaCl_2$.

In other experiments, not described here, we did not see any appreciable influence of the presence of aluminum ions on the setting temperature, which is in agreement with the findings of Hinton *(13)*.

From our results with pectins having degrees of esterification of 53% and 75% we may conclude that a large increase of pectin concentration causes only a small rise of setting temperature.

Hinton *(13)* found a lack of correlation between changes in setting temperature and jelly strength. We see also that the setting temperature is decreasing in the pH range from about 2.0 to 2.6 where for all pectins a constant gel strength exists (see Figure 2). Nevertheless, it is clear that the sudden fall of setting temperature at a

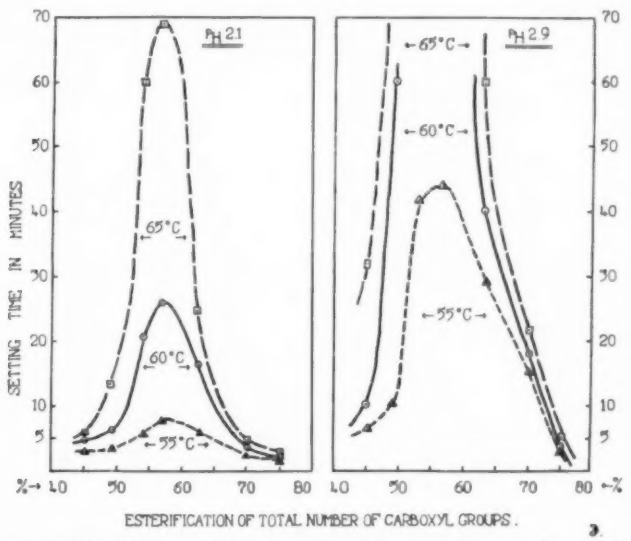

Figure 5. Setting times of jellies at pH 2.1 and pH 2.9 from pectins with different degree of esterification, during rapid cooling in a waterbath to 55° C, 60° C or 65° C.

higher pH than about 2.8, shown in Figure 4, only by jellies made from the pectin with 52% esterification without $CaCl_2$ or only with the addition of apple juice, is connected with the decrease of jellying power under these circumstances as shown in Figure 2. Further it is stated that aluminum has no influence on setting temperature, but it is known that it causes an important increase of jelly strength *(7, 13)*.

Setting time of jellies made from pectins with different degrees of esterification after rapid cooling to constant temperatures. For these experiments, the test tubes

with jelly mixture were placed immediately after filling into a vigorously stirred waterbath at constant temperature. The time required for the mixture in the tubes to reach the temperature of the waterbath was noted. When the temperature of the waterbath was 65° C, 60° C, or 55° C, the times were respectively about 4¼, 4¾ and 5 min.

Results of trials with jellies from pectins with different degrees of esterification and at pH $2.1 \pm 0,05$ and pH $2.9 \pm 0,05$ are shown in Figure 5.

We see that the setting time was greatly influenced by the degree of esterification and that relatively short setting times were found for jellies with pectins having degree of esterification higher than about 70% and lower than about 50%.

It is clear that setting time is increased by a rise of pH of the jellies as it has been mentioned by other authors *(7, 14, 15)*. It is remarkable that for different jellies rather long setting times have been noted (70 min), which are easily reproducible with an accuracy of ± 1 min.

Influence of jelly grade on setting time. A series of pectins with diminishing jelly grade were used for the experiments concerning the influence of jelly grade of pectin on the setting time of jellies. These were prepared from apple pectin by (a) enzymatic breakdown, (b) thermal degradation, (c) decomposition by H_2O_2 and ascorbic acid.

Jellies were made from each pectin at several different acidities. The setting time tubes were cooled in air and the extent of cooling was measured by thermocouples. During the rapid cooling in air some undercooling of the jellies may have occurred, especially in the jellies with higher pH values.

In Figures 6, 7, or 8 the setting time of jellies from pectins with various jelly grades is plotted against the pH of the jellies. At the setting time axis the mean temperatures are given which were noted in the tubes after different times.

In all cases a lower jelly grade of pectin is related to a longer setting time of the jellies, but this phenomenon is more pronounced for the pectin series prepared by thermal degradation or action of H_2O_2 and ascorbic acid than for the pectins prepared by enzymic breakdown. Without repeating these trials it is difficult to say if this difference in the results is typical for the particular method of depolymerization used.

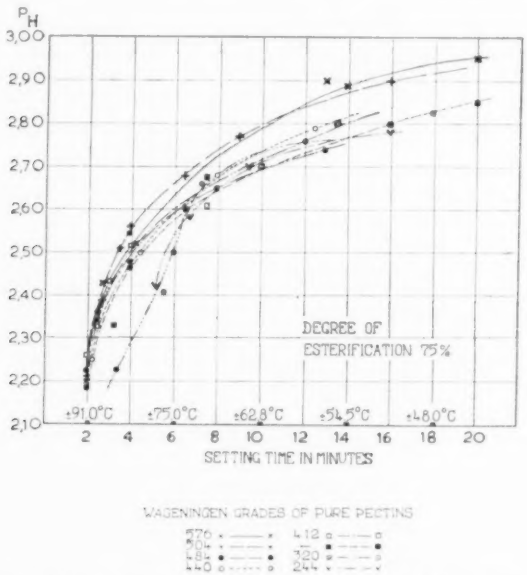

Figure 6. Relation between setting time and pH of jellies from pectins with different jelly grade, prepared by enzymatical decomposition.

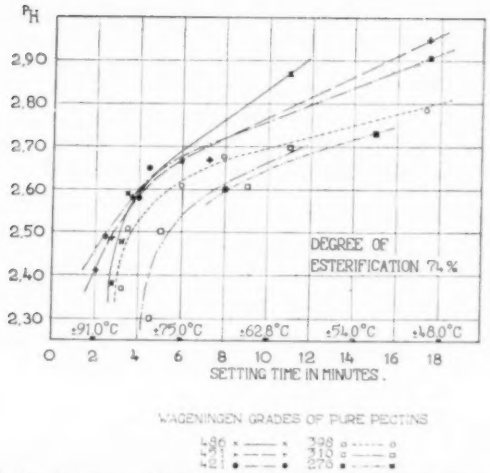

Figure 7. Relation between setting time and pH of jellies from pectins with different jelly grade, prepared by thermal breakdown.

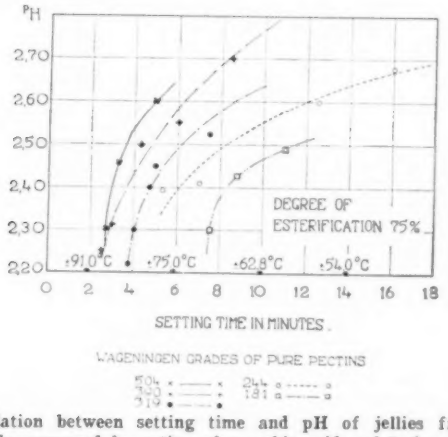

Figure 8. Relation between setting time and pH of jellies from pectins with different jelly grade, prepared by action of ascorbic acid and hydrogen peroxide.

In the foregoing experiments, pectins have been used with a great variation of jelly grade, but as Christensen *(3)* has shown, the molecular weights of commercial pectins differ by as much as 90,000. Olsen, Stuewer, Fehlberg and Beach *(18)* reported that molecular weights of about 40,000, 100,000, and 175,000, respectivey, correspond with poor, moderate, and very good jellying powers.

DISCUSSION

Some rather long setting times have been found for jellies after rapid cooling to a constant temperature (Fig. 5), and it is evident that with decrease of temperature a shortening of setting times occurs. In other trials,

not reported here, we also found long setting times for pectins with a high degree of esterification when cooling to 90° C or 80° C.

It seems reasonable to conclude that a jelly mixture shows a different velocity of the setting "reaction" at different temperatures, infinitely slow at relatively high temperatures and increasing with decrease of temperature. Within a certain set of conditions (sugar content, pH, ash constituents), the level of temperature range at which the setting time changes from infinitely long to a short period is strongly influenced by the degree of esterification of the pectin used.

During cooling at a certain rate, jellification starts at a certain temperature and develops faster at lower temperatures. At a slow cooling rate the setting "reaction" has the opportunity to proceed further at higher temperatures than with rapid cooling.

In other words, with slow cooling the integration of the setting "reaction" during the longer period at relatively high temperatures causes setting into a coherent mass at higher temperature than during rapid cooling. In this way it is possible to explain the "undercooling" found in Hinton *(13)*.

In Figure 3 it is shown that these "undercooling" effects increase with rise of the pH of the jelly mixture. From these facts it is concluded that at higher pH values there should be a relatively slow increase of velocity of jellification with decrease of temperature, whereas at low pH values the change of setting velocity from infinitely slow to very rapid should occur within a narrow range of temperatures.

From the reported results it is impossible to conclude at which temperature the formation of a jelly is starting.

Therefore it should be necessary to measure setting times during very long periods and in many cases it has to be expected that under these conditions partial decomposition of the pectin is occurring. Also it is not known if the same types of bondings are involved in the earlier and later steps of jellification. For these reasons it is doubtful if it is possible to indicate accurately for any set of conditions the highest temperatures at which jellification into a coherent mass can be completed. Therefore, it is also questionable if a real sharp setting temperature as a determinate physical property may be found, as is indicated by Hinton *(13)*. Moreover, Hinton has found, within limits of accuracy, the same setting temperature and re-melting temperature for sugar-acid-pectin jellies. However, in our experiments, in no case was the re-melting of the jellies succeeded at the measured temperature of setting (results from Figures 3 and 4), but in all cases these jellies have resisted heating to 100° C.

Nevertheless, for practical purposes, the measurements of setting temperatures may be very useful, but this is also true for the determinations of setting times.

The mechanism of jellification has been discussed by various authors *(7, 11, 12, 17)* on the basis of precipitation of pectin under the prevailing conditions of jelly formation, whereas a uniform dispersion of pectin molecules is maintained. According to these views, the different possibilities for the setting of jellies at various temperatures should be explained as a different solubility of the pectin in the jelly mixture at these temperatures. In connection with this hypothesis, the results of experiments about setting time or setting temperature indicate that decrease of solubility of pectins at higher

temperatures should be caused by lowering of pH, increase of degree of esterification and of jelly grade, and by ash constituents such as calcium. The more the esterification of pectin is decreased the stronger is the influence of such ash constituents as calcium.

Experiments with cooling jellies to a constant temperature employed pectins with had been purified by two-fold precipitation in 70% acetone at pH 1.5 followed by washing in pure acetone. Various authors have stated, however, that it is rather difficult to remove the last traces of ash by precipitation or dialysis *(10, 18, 19)*, especially iron and aluminum.

It may be that the shortening of setting times which has been reported for pectins with a rather low degree of esterification (Figure 5), can be explained by the lower solubility of these pectins in acid environment, and also by the susceptibility of the solubility of these pectins to traces of ash constituents from the pectin or the sugar. It is known that in some cases low concentrations of iron and aluminum have a considerable effect on jellification *(7, 13)*.

From Figure 4 it may be concluded that the setting temperature is rather low at pH values in the neighborhood of the maximum pH of jellying. The temperature after cooling is not much lower than the temperatures at which the setting time is expected to be very long. From this fact it can be understood that the stiffening of jellies may take a very long time in gels with marginal pH for jellification *(16)*.

A strong increase of pectin concentration causes only a very limited rise of setting temperature. For the maintenance of the same maximum jelly strength, when comparing the setting times of jellies from pectins with different jelly grade, it has been necessary to use much higher concentrations of the more decomposed pectins (Figures 6, 7 and 8). Nevertheless, it is obvious that the decrease of setting temperature, caused by diminished jelly grades, supersedes the small rises of setting temperatures resulting from increasing amounts of pectin.

It is important to use pectins with a high degree of polymerisation for commercial rapid set preparations. In our studies about jelly grade of apple pectins *(8)* nearly the same jelly grades of pectins have been found in fruits from different varieties and some unpublished data show the same for apples of different quality.

Therefore, it seems reasonable to suppose that the difference in jelly grade of commercial apple pectins *(3)* is caused by the use of different extraction methods or mainly is due to influences during the manufacture and storage of apple pomace *(9)*. It is well known that after pressing the apples a rather rapid decomposition of pectins may occur by fungal enzymes *(2)*; in other causes perhaps oxidative reactions may exert such an influence *(6)*.

In Figure 2 it is shown that the jellying power of a pectin is rather strongly increased by partial saponification in acid environment (pH 1,0, 60° C), whereas under these conditions no appreciable diminishing of clain length of pectin molecules or molecular weight occurs *(7)*. This rise of jellying power is in contrast with the narrow relationship between molecular weight (measured by viscosity) and jellying power, which was found by Christensen *(3)* in his investigation concerning the relation of molecular weight and jelly grade.

SUMMARY

A study has been made of the different factors governing the setting time or setting temperature of sugar-acid-pectin jellies, especially the influence of jelly grade, degree of esterification and concentration of the pectins in the jellies and the influence of ash constituents.

A theory to explain the "undercooling" of jellies during rapid cooling has been given.

For the study of the influence of jelly grade, pectins with various degrees of polymerization have been prepared by enzymic and thermal decomposition and by the combined action of ascorbic acid and hydrogen peroxide. The partial breakdown by the non-enzymic treatments caused a considerable increase of setting time of the jellies.

The influence of the degree of esterification has been studied by using pectins with different degrees of esterification prepared by saponification in acid environment. The setting time of jellies containing pectins with a low ash content increases with decrease of degree of esterification of pectins to about 50% while jellies from pectins with lower degrees of esterification exhibit shorter setting times.

It has been found that the addition of calcium to jellies causes an increase of setting temperatures in all cases, but this effect is much stronger when the pectins have lower degrees of esterification. Therefore, pectins with a degree of esterification of about 45%-55% can be used as rapid set pectins.

The jellying power of partially saponified pectins is considerably above the jellying power of the pectins with high degree of esterification from which they were prepared.

Acknowledgment

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STUDIES ON CANNED CHOCOLATE NUT ROLL EXPERIMENTALLY INOCULATED WITH SPORES OF *CLOSTRIDIUM BOTULINUM* TYPES A AND B[a, b]

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Chocolate nut roll is a high calorie product having a very satisfactory flavor acceptance rating; hence, it is desirable that it be included in certain military rations. This non-sterile, steam processed item has a pH above 6.0, which, together with a moisture level of 28 to 29%, places it in the category of a food that may be a potential botulism hazard when vacuum packed in cans. Furthermore, data regarding the possible inhibitory effect of sucrose on the growth and toxin production of *Clostridium botulinum* in this product are not available in the literature. Therefore it was important to conduct experiments on representative samples to determine moisture, pH and sucrose levels which will eliminate the botulism hazard. The objectives of this study were: (1) to determine whether canned steamed chocolate nut roll, of the composition desired for inclusion in military rations, is safe from the botulism hazard, and if so (2) to establish approximately how much the composition could vary (e.g. an increase in moisture or pH, or a decrease in sucrose) without yielding a medium satisfactory for growth and toxin production of *C. botulinum*.

Data from two series of experiments are presented and discussed in this paper. In the first series samples were prepared to test the safety of canned chocolate nut roll at moisture levels near 29% in the presence of 0 and 27% sucrose, and at moisture levels of approximately 40% in the presence of 0, 23, 27, and 31% sucrose. The actual moisture levels, as determined by analysis, were somewhat higher than had been anticipated. In the second series samples were set up to include chocolate nut rolls with moisture levels near 29% in the presence of 0 and 27% sucrose, and with moisture levels of approximately 36% in the presence of 0, 20, 25, and 30% sucrose. Spores of six strains of *C. botulinum* (three each of types A and B) shown previously as having varying resistances to heat were used as the inoculum. In addition, several uninoculated control cans were included with each experimental series.

MATERIALS AND METHODS

Approximately 1,000 seven-ounce cans of chocolate nut roll were prepared, canned, steamed, sealed and stored at the Quartermaster Food and Container Institute for the

[a] The experiments reported in this investigation were carried out in cooperation with the staff of the Quartermaster Food and Container Institute for the Armed Forces.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 1031 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

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Armed Forces in Chicago by members of the Institute staff. The nut roll of normal composition had the following formula (in the other lots sugar was omitted or additional moisture was incorporated):

Ingredients	Weight in Ounces
Sugar	27.0
Flour[1]	20.0
Water	22.0
Pecans	8.0
Bitter Chocolate	7.0
Eggs	7.0
Shortening[2]	6.0
Milk[3]	2.0
Salt	0.5
Vanilla	0.5

[1] Flour: low protein, short patent, hard winter wheat.
[2] Shortening: hydrogenated shortening, 100 hour stability.
[3] Milk: non-fat dry milk solids.

A sufficient volume of a phosphate buffer spore suspension containing equal numbers of types A and B, *C. botulinum* (3 strains of each) was inoculated into each of the batches to give an inoculum of 700,000 spores per can (7 oz of batter).

The following strains of *C. botulinum* were used in the inoculum:

Strain	Type	Obtained from
457	A	Dr. L. S. McClung, University of Indiana
62A	A	Dr. K. F. Meyer, Hooper Foundation, University of California
109	A	Dr. K. F. Meyer, Hooper Foundation, University of California
169	B	Dr. K. F. Meyer, Hooper Foundation, University of California
12033	B	National Canners Association
126.7	B	Department of Microbiology, University of Chicago

The uninoculated chocolate nut roll samples (lots 1-6 and 13-18) were prepared first to minimize the possibility of contamination with spores of *C. botulinum*. The nut roll was steamed at 212° F for 3 hours. Immediately upon removal from the steamer the cans were sealed, stacked one deep on racks and then air cooled. The sealing operation was made on pilot plant sealing equipment. Before opening a can an absolute vacuum reading was taken.

All samples were stored at 90° F and were cycled weekly at 40° F for 16 hr. This cycling was done to simulate practical storage conditions favoring condensation of moisture in portions of the can.

At the end of each storage period one can was selected at random from each group to provide a sample for pH analysis. The pH determinations were made on 15 g nut roll suspended in 100 ml of freshly boiled and cooled distilled water. A Beckman potentiometer, Model H2, was used for these analyses.

Three loaves from each uninoculated and 10 loaves from each inoculated group were analyzed for appearance, consistency, odor and toxin as soon as practicable after preparation. Similar numbers of cans from each group were examined after 1 and 2 weeks, and after 1, 3, 6, and 12 months of storage at 90° F (in the first series of experiments, only nut roll with no added sucrose was tested after 1 and 2 weeks of storage; in the second series, no samples were analyzed after 1 week and only nut roll with no added sucrose was tested after 2 weeks). One can from each uninoculated and 2 cans from each inoculated group were analyzed for the presence of viable spores at each of these intervals. All nut roll cans and samples were stored in the refrigerator until needed.

Moisture analyses on representative samples from the contents of 2 cans of each uninoculated and 3 cans of each inoculated group were conducted according to the vacuum drying method specified in *Cereal Laboratory Methods (1)*. Uninoculated samples (lots 1-6) from the first series were not analyzed for moisture.

After the top of each can had been cleaned with alcohol and flamed, the end with the headspace was opened (cans from which samples for spore recovery analyses were to be obtained were opened under a hood using aseptic technique). Can openers were thoroughly flamed before using. Each loaf was observed for appearance, consistency, and odor. The nut roll was mixed with a sterile wooden tongue depressor during the addition of 200 ml of sterile 0.85% saline (making a 1:2 dilution of the nut roll). The can was then covered with a sterile Petri dish top or bottom. The following day 20 ml portions of the fluid from the settled nut roll suspensions were transferred to sterile centrifuge tubes. After centrifuging these samples at high speed, the clear supernatant fluids were used for mouse inoculations. Test mice were injected intraperitoneally with 0.7 ml of this fluid, and were observed for 4 days. Where a mouse died, a series of 4 mice was inoculated. All mice received 0.7 ml of the toxic sample. One of these mice received in addition 0.1 ml of type A, a second 0.1 ml of type B and a third 0.3 ml of polyvalent ABC antitoxin. The fourth received no antitoxin.

Ten ml portions of the nut roll suspension from one can of each control group (1 can of 3) and from 2 cans of each inoculated group (2 cans of 10) were pipetted to flasks containing 90 ml of beef heart medium for determination of the presence of viable *C. botulinum* spores. Decimal dilutions of the inoculated nut roll suspensions were also made in tubes containing 9 ml of beef heart medium to estimate the approximate number of viable spores. The inoculated beef heart flasks and tubes were heated to 80° C for 10 min to destroy any viable vegetative cells and were then incubated at 30° C for 10 days. Following incubation the clear centrifuged supernatant fluid from the cultures was tested for toxicity in mice to determine whether any viable spores had survived steaming and storage. Each mouse received, by intraperitoneal injection, 0.5 ml of the broth. Where a test mouse died a series of 4 mice was injected as described above, using a 1:10 saline dilution of the toxic sample as the test fluid.

There were no samples available from lots 9 and 10 for analysis after 6 and 12 months of storage, because all remaining cans from these groups were moved from the incubator to the refrigerator at the end of 3 months because of swelling.

RESULTS

Vacuum readings. Vacuum readings on the chocolate nut roll samples varied considerably. The initial samples had readings varying from 0 to 27 in with most of them in the range 12 to 19 in. In general, it was found that with an increase in storage time, there was an increase in the number of cans having reduced vacuum, both in the inoculated and uninoculated groups.

Among the samples in the first series (lots 1-12), it was observed that cans which contained toxin usually had a decreased vacuum. Some of the inoculated samples with high moisture content showed positive pressure in the cans after 2 weeks' storage. After 12 months' storage vacuum readings on samples at the lower moisture levels varied from 28 lb pressure to 27 in vacuum with the majority in the range from 2 lb pressure to 23 in vacuum. Cans containing the higher moisture samples had readings from 27 lb pressure to 1 in vacuum with most of them in the range from 2 to 14 lb pressure.

In general, cans of chocolate nut roll from the second series (lots 13-24) maintained their vacuum better than was observed with the first series. Five cans in these groups had no vacuum or showed a positive pressure. After one month of storage one can of lot 17 had 4 lb pressure, and one each from lots 22 and 23 had 0.5 lb pressure. After 3 months one can from lot 17, and after 6 months one can from lot 15, had no vacuum. Readings on the samples analyzed after 12 months of storage varied from 6 to 24 in of vacuum, with the majority in the range 13 to 20 in.

Appearance, consistency and odor. One of the cans of chocolate nut roll examined initially and after 2 weeks of storage at 90° F, only those of lots 2, 8, 14, and 20 had the characteristic appearance and consistency desired. All other samples did not fill the cans upon steaming. Samples of lots 1, 7, 13, and 19 were dry and hard. Those of lots 3 to 6 and 9 to 12 were usually very moist and fudgelike. Cans with no sugar and a high moisture level (lots 3, 9, 15, and 21) had a slight fermentation odor after 2 weeks of storage. After 12 months of storage the contents of cans at the lower moisture levels had not changed in appearance from that observed at the earlier analyses. Samples in the groups containing sugar and having moisture levels in excess of 35% were moist and fudgelike, and some separation of a syrupy liquid was observed. In a few of the

lower moisture samples slight fermentation odors were noted; in most of the high moisture samples strong fermentation odors were encountered. The odors from the uninoculated samples were in most instances only slightly less intense than those from comparable inoculated cans. In general, a fermentation odor was accompanied by a drop in vacuum.

pH. The pH readings on the contents of representative cans, taken at the initial analysis (no storage at 90° F) and on similar samples taken after 3 and 12 months of storage at 90° F are shown in Table 1. The larger changes occurred among the higher moisture samples exhibiting definite fermentation.

With most lots of chocolate nut roll tested in the first series (lots 1-12), the pH readings after 12 months were 0.2 to 2.1 lower than the corresponding initial values; in the second series (lots 13-24) the final readings were mostly 0.1 to 0.4 lower. There was a marked difference in the initial pH readings of the second experiment as compared to those of the first nut roll experiment. This difference was probably due to the higher acidity of the chocolate syrup used in preparing the batters for the second experiment.

Moisture. Moisture determinations were made on two cans from each uninoculated and on three cans from each inoculated lot (moisture determinations were not made on uninoculated nut roll of lots 1-6). A mean moisture level for each lot was calculated from the results of these analyses and the individual values are also listed to give the probable range of variation among the samples (see Table 2).

Botulinum toxin. Toxin was found in approximately one-third of the inoculated samples of the first experimental series (lots 7-12). Most of the positive samples were from the groups having moisture levels in excess of 42%. All cans of chocolate nut roll of the composition desired for rations (27% sucrose; 29.41 to 29.95% moisture) were negative for botulinum toxin at all analyses up to and including 12 months of

TABLE 1

pH of canned chocolate nut roll after three storage intervals at 90° F

Lot No.	Percent sucrose	Spore inoculum per can	Initial	Storage time	
				3 months	12 months
1	0	None	6.9	6.8	6.4
2	27	None	6.8	6.8	6.8
3	0	None	7.2	5.8	5.1
4	23	None	6.8	6.5	5.8
5	27	None	6.7	6.5	5.6
6	31	None	6.8	6.3	5.9
7	0	700,000	6.8	6.5	6.6
8	27	700,000	7.1	6.7	6.8
9	0	700,000	6.8	6.0	Not done
10	23	700,000	7.0	6.4	Not done
11	27	700,000	7.1	6.4	6.1
12	31	700,000	7.4	6.4	6.3
13	0	None	6.2	6.1	5.9
14	27	None	6.2	6.3	6.0
15	0	None	6.1	6.1	6.1
16	20	None	6.2	6.0	5.9
17	25	None	6.1	6.1	5.7
18	30	None	6.1	6.0	5.7
19	0	700,000	6.0	5.8	5.6[1]
20	27	700,000	6.0	6.0	5.8
21	0	700,000	5.9	6.0	5.8[1]
22	20	700,000	6.0	6.0	6.0
23	25	700,000	5.8	6.2	6.0
24	30	700,000	5.9	6.1	5.8

[1] The cans from which these samples were taken were incubated 15 months at 90° F.

TABLE 2

Moisture analyses on representative cans of chocolate nut roll used in these studies

Lot No.	Percent moisture individual samples			Percent moisture average
	A	B	C	
7	35.92	35.83	35.73	35.83
8	29.58	29.41	29.95	29.65
9	45.19	45.50	45.95	45.55
10	42.73	43.47	44.28	43.49
11	43.22	42.13	42.78	42.71
12	44.11	44.90	44.07	44.36
13	29.01	29.65		29.33
14	30.42	30.02		30.22
15	38.22	38.40		38.31
16	36.26	36.30		36.28
17	37.80	37.18		37.49
18	38.23	39.08		38.65
19	30.00	29.76	30.31	30.02
20	30.07	29.40	29.32	29.60
21	39.74	38.94	39.17	39.28
22	35.67	36.88	37.01	36.52
23	34.70	35.33	36.38	35.47
24	37.49	37.13	37.33	37.32

storage at 90° F. No toxic samples were found in group 11 at the final analysis. The contents of several cans in this group were positive after 1, 3, and 6 months of storage. All uninoculated control samples were negative for toxin. The results of the toxin analyses of the inoculated samples are summarized in Table 3.

In the second nut roll experiment (lots 13-24), 3 uninoculated and 10 inoculated samples were tested for toxin initially and after storage at 90° F for 2 weeks and 1, 3, 6, and 12 months. All samples were negative for botulinum toxin.

Viable spore count. Type A botulinum toxin was produced in beef heart medium from a 10 ml inoculum of a 1:2 saline dilution from one inoculated can of lot 3 at the 6-month analysis. This was the only positive sample from the uninoculated groups encountered in these experiments.

Analyses for viable *C. botulinum* spores present in representative samples of inoculated steamed chocolate nut roll were conducted at each interval that nut roll was tested for botulinum toxin. Results of analyses for viable *C. botulinum* spores in samples tested initially and after 3 and 12 months of storage at 90° F are shown in Table 4.

TABLE 3

Toxin analyses of canned chocolate nut roll after various storage intervals at 90° F

Lot No.	Percent moisture average	Percent sucrose	None	No. of cans containing botulinum toxin after storage for:[1]					
				1 wk	2 wks	1 mo	3 mos	6 mos	12 mos
7	35.83	0	0	1[2]	1	4	7	5	9
8	29.65	27	0			0	0	0	0
9	45.55	0	0	5	9[3]	9	10	Not done	Not done
10	43.49	23	0			3	1	Not done	Not done
11	42.71	27	0			6	4	3	0
12	44.36	31	0			0	0	0	0

[1] Ten cans of each lot were analyzed at each interval.
[2] Only samples with no sugar were analyzed at 1 and 2 weeks.
[3] Only 9 cans of this lot were available for analysis.

TABLE 4

Presence of viable spores of Clostridium botulinum, types A and B, in canned chocolate nut roll after steaming at 212° F for 3 hours (1:2 dilution of chocolate nut roll)

Lot No.	No. of cans in which viable spores were present in:[1]				
Sample: / Spores:	10 ml 2 or less	1.0 ml 2-20	0.1 ml 20-200	0.01 ml 200-2000	0.001 ml 2000-20,000
Initial analyses					
7	1	0	0	0	0
8	2	1	0	0	0
9	0	0	0	0	0
10	0	0	0	0	0
11	2	0	0	0	0
12	0	0	0	0	0
19	0	0	0	0	0
20	2	2	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
Three month samples					
7	2	2	2	1	1
8	2	0	0	0	0
9	2	2	2	2	2
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
19	0	0	0	0	0
20	0	0	0	0	0
21	0	0	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
Twelve month samples					
7	2	2	2	2	1
8	0	0	0	0	0
9			Not done		
10			Not done		
11	0	0	0	0	0
12	0	0	0	0	0
19	0[2]	0[2]	0[2]	0[2]	0[2]
20	1	0	0	0	0
21	0[2]	0[2]	0[2]	0[2]	0[2]
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0

[1] Cans were tested in duplicate.
[2] The cans from which these samples were taken were incubated 15 months at 90° F.

These data indicated that few viable spores remained. The higher numbers observed in random samples at some analysis intervals from groups 7 and 9 probably resulted from growth of *C. botulinum* in the product. There were no samples available from lots 9 and 10 after 6 and 12 months of storage, because all remaining cans from

these groups were moved from the incubator to the refrigerator at the end of 3 months' storage because of excessive swelling. The number of spores added to all inoculated samples was approximately 3,500 per g.

DISCUSSION

Inoculated chocolate nut roll used in the first experiment (lots 7-12) had initial pH levels within the range 6.8 to 7.4. Most of the toxic samples were from the groups having moisture levels in excess of 42%. The inoculated nut roll used in the second experiment (lots 19-24) had initial pH levels within the range 5.8 to 6.0. There was a marked difference in the initial pH readings in this experiment as compared to those of the first nut roll experiment. This difference was probably due to the higher acidity of the chocolate syrup used in preparing the batters for the second experiment. Consequently, the data from the second experiment will not in all cases be directly comparable to that of the first experiment. Special precautions should be observed in comparing toxin production results.

The reason for the absence of growth of *C. botulinum* in the second experiment as compared to the good growth obtained in the first experiment was not apparent. The pH of the canned chocolate nut roll in the second experiment was lower than that in the first (5.8 to 6.0 as compared to 6.8 to 7.4) which is not a limiting factor to the growth of *C. botulinum*. Of the basic ingredients in chocolate nut roll, chocolate is subject to the most variation. One batch of chocolate may contain inhibiting substances for *C. botulinum* that may be absent in another batch. For example, one component of chocolate is oxalic acid. Some thought was given to its role as an inhibitory agent, but since no methods were available for detecting oxalic acid in food this factor was not studied. The pH of chocolate may also influence bactericidal properties to *C. botulinum* since a pH suitable to the growth of *C. botulinum* (6.0) in a favorable medium was inhibitory to growth in chocolate nut roll.

Data obtained in previous experiments designed to test the safety of canned bread *(3)* showed that with bread initially adjusted to pH levels near 4.8 no toxin was produced in simples inoculated with *C. botulinum* even at moisture levels near 40%. In similar bread initially adjusted to approximately pH 5.8 toxin was produced after 12 months of storage at 90° F in all samples with moisture levels above 36%.

Segalove and Dack *(2)* observed in the case of dehydrated meat that growth and toxin production of *C. botulinum* occurred when the moisture content was 40% but not 30%.

Viable spores were present in many of the inoculated cans even after storage for one year at 90° F; hence, safety is assured only if the chocolate nut roll continues to be inhibitory to growth and toxin production of *C. botulinum*.

SUMMARY

A study was made to assess the botulism hazard of canned steamed chocolate nut roll of the composition desired for inclusion in military rations. Analytical determinations were made of the effect of varying concentrations of sucrose on growth and toxin production of *Clostridium botulinum*.

Twenty-four lots of chocolate nut roll were prepared, steamed at 212° F for 3 hours and vacuum packed in cans. All samples were stored at 90° F

and were cycled weekly for 16 hr at 40° F for intervals up to 12 months. In the first series of experiments (lots 1-12), samples were prepared to test the safety of canned chocolate nut roll at moisture levels near 29% in the presence of 0 and 27% sucrose, and at moisture levels of approximately 40% in the presence of 0, 23, 27, and 31% sucrose. The actual moisture levels, as determined by analysis, were somewhat higher than had been anticipated. In the second series (lots 13-24), samples were set up to include chocolate nut rolls with moisture levels near 29% in the presence of 0 and 27% sucrose, and with moisture levels of approximately 36% in the presence of 0, 20, 25, and 30% sucrose.

In the first experiment, toxin was found in approximately one-third of the inoculated samples. Most of the positive samples were from groups having moisture levels in excess of 42%. All cans of chocolate nut roll of the composition usable for rations (27% sucrose; 29.41 to 29.95% moisture) were negative for botulinum toxin at all analyses up to and including 12 months of storage at 90° F. In the second experiment, toxicity tests on samples analyzed initially, after 2 weeks, and 1, 3, 6, and 12 months of storage showed that in inoculated canned chocolate nut roll having initial pH reading in the range 5.8 to 6.0, moisture levels of 39.75% or lower were limiting to growth and toxin production of *C. botulinum* at all sucrose levels tested.

Analyses for the presence of viable *C. botulinum* spores after storage of chocolate nut roll for 12 months at 90° F. showed that the probable number remaining in most samples was 2 or less per gram. The higher numbers observed in some groups of samples probably resulted from growth of *C. botulinum* in the product.

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MAPLE SIRUP. XIV. ULTRAVIOLET IRRADIATION EFFECTS ON THE GROWTH OF SOME BACTERIA AND YEASTS[a]

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Collection of maple sap has undergone drastic changes in the last few years. The conventional spout and bucket system is rapidly being replaced by transparent plastic bags and, more recently, by plastic tubing capable of transporting sap directly from trees to collection tanks.

Maple sap is susceptible to microbial infection, which, when it occurs, lowers the quality of the syrup. Recent studies in this laboratory *(14, 18)* have shown that sufficient ultraviolet radiation from sunlight is transmitted through plastic bags and tubing to cause a reduction in microbial populations of naturally-contaminated and inoculated maple sap. The advantages of a collection system which permits entrance of sunlight are apparent. It was considered that the use of germicidal lamps for the disinfection of contaminated sap and for keeping stored sap sterile might have even greater potentials in the control of microbial infection.

Since the first observations on the germicidal action of sunlight by Downes and Blunt *(12, 13)*, the effects of solar radiation have been reported for a variety of organisms *(2, 3, 22, 24, 25)*. It is generally acknowledged that the most lethal portion of the sunlight spectrum is in the vicinity of 260-270 mmc *(6, 7, 9, 17)*. The germicidal effect *(4, 5, 9, 20, 21)* as well as the mutagenic effect *(23, 26)* of ultraviolet radiation have been reported by several workers.

The beneficial effect of mild heating or slightly elevated temperatures during post-irradiation treatment has been observed by several workers *(8, 11, 16)*. Data are not available, however, comparing recovery rates at low and moderate incubation temperatures.

Extensive studies have not been made comparing the effectiveness of a single dosage of radiation with an equal dosage given in several periods of shorter exposure. Coblentz and Fulton *(9)* found little if any differences in the survival rates of *Bacterium coli communis* exposed to radiation continuously or intermittently with short and long intervals of rest.

The influence of flow rate of a liquid menstruum during exposure to ultraviolet radiation was investigated by Cortelyou *et al (10)*. As anticipated, decreased destruction rates were observed in faster moving fluids.

The purpose of this investigation was to study the effect of ultraviolet radiation on microorganisms in maple sap. The variables tested were chosen from conditions which prevail naturally or which are controllable during sap collection. Representative strains, isolated from naturally-contaminated

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sap, were employed as the test organisms. These included one yeast and two bacterial strains employed in previous studies *(15, 19)*.

In the sugar bush sunlight may exert a continuous effect throughout the daylight hours, or may be discontinuous and even variable in intensity due to intervening night hours, cloudy skies or shadows cast by trees. Previous work in this laboratory *(18)* had suggested that intermittent exposure to sunlight, during periods of low temperature, caused a greater destructive effect than continuous radiation of similar total dosage. These variable conditions were simulated by use of several dosage intensities and by use of continuous and discontinuous methods of exposure to ultraviolet radiation.

The temperature of maple sap in the sugar bush, during or following sunlight radiation, may vary from near-freezing to moderate. It was important to know whether or not temperature exerted any beneficial or detrimental effect on the recovery and subsequent growth of irradiated cells. Consequently, two incubation temperatures, 41° F and 80.6° F, were employed in the estimation of survivors.

The possibility was investigated of employing a commercial germicidal (ultraviolet) lamp to suppress microbial activity in large quantities of stored sap and to disinfect contaminated sap during handling. For the latter, sap was run over a glass-jacketed germicidal lamp to evaluate the effectiveness of ultraviolet radiation as a disinfectant on flowing sap.

All experiments were designed to permit statistical evaluation of the data so that an unbiased appraisal could be made.

MATERIALS AND METHODS

A. Apparatus and Equipment

1. Ultraviolet source for static system. General Electric 15- and 30-watt, 110 volt germicidal lamps mounted in reflecting fixtures.
2. Ultraviolet source for flowing system. General Electric 30-watt, 110 volt germicidal lamp encased by a concentric glass tube with approximately 0.5 cm space between lamp and tube as shown in Figure 1.
3. Coleman model 14 spectrophotometer.
4. Sterile maple sap, collected and stored in 1-gallon cans at 5° F as described previously *(15)*, was thawed at room temperature and transferred aseptically to sterile flasks.

B. Organisms

Typical isolates from naturally-contaminated maple sap were used.

Pseudomonas-25 (deposited in the Northern Regional Research Laboratory collection and labeled NRRL B-1890 and *Pseudomonas*-11 (NRRL B-1888) were grown in petri plates on tryptone-glucose-yeast extract (TGY) agar for 24 hr at 80.6° F. Surface growth was removed by washing and diluted with sterile sap to obtain the desired concentration. An approximate cell concentration was estimated spectrophotometrically by comparison of the optical density of the diluted suspension with a standard turbidity curve prepared from suspensions of known concentration. Final estimations of the cell concentration were always made by plating and colony counts.

Cryptococus albidus (NRRL Y-2422) suspensions were prepared in a similar manner from 48 hr cultures grown on Sabouraud agar (adjusted to pH 4.5 with sterile 10% lactic acid) at 80.6° F.

C. Irradiation procedure (static)

Pseudomonas-25. The stock suspension was diluted with sterile sap to a concentration of 1.4×10^8 organisms per ml. Fifteen ml of inoculated sap were transferred to each of

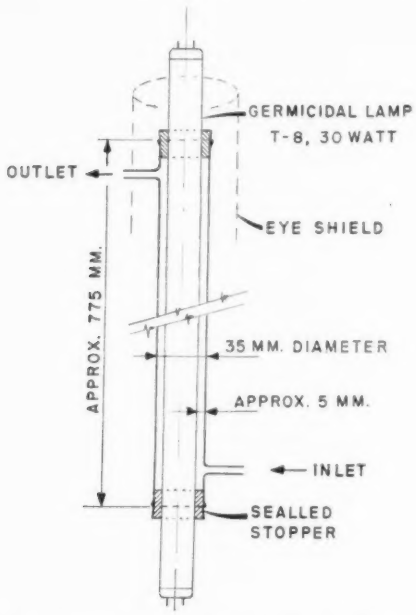

Figure 1. Apparatus used for ultraviolet irrdaiation of cells suspended in a moving liquid medium.

12 petri plates (3 sets of 4) and subsequently exposed to ultraviolet radiation as described below. The 15-watt Germicidal Lamp was mounted 100 cm above and parallel to the bench surface. All plates were placed in the same position relative to the lamp during exposure. Petri plate covers were removed prior to irradiation and then replaced immediately following exposure. The following methods of exposure were employed.

1. *Continuous irradiations.* Exposure times for this set of plates were 15, 30, 45 and 60 sec, respectively. Immediately following irradiation, the suspensions were diluted and plated with TGY agar. The plates were incubated at two recovery temperatures. Colonies were counted after 3 days at 80.6° F and after 10 days at 41° F. Triplicate plate counts were employed for all dilutions and for both incubation temperatures.

The second set of 4 plates containing inoculated sap was irradiated as described above and then held for 5 hr at 41° F, prior to dilution, plating, and incubation at the two temperatures employed.

2. *Discontinuous irradiations.* Irradiation of the third set of plates consisted of three exposures separated by two holding periods of 2½ hr each at 41° F; each exposure per plate was 1/3 the total time for the corresponding plates irradiated by the continuous method. Following the third exposure period, the suspensions were appropriately diluted, plated and incubated at 41° F and 80.6° F.

All plates were held in the dark at 41° F except during radiation and plating to minimize photoreactivation and possible stimulation of recovery and/or growth by elevated temperatures.

Pseudomonas-11. A suspension containing 1.2×10^8 organisms per ml was prepared and irradiated as described for *Pseudomonas*-25.

Cryptococcus alibidus. A suspension containing 2.3×10^7 organisms per ml was prepared and irradiated by the continuous and discontinuous methods with a 30-watt

germidical lamp mounted 35 cm from the bench surface. Survivors were grown on acidified Sabouraud agar, and after incubation periods of 4 days at 80.6° F and 10 days at 41° F colony counts were made.

D. Irradiation procedure (flowing)

Three other suspensions of *C. albidus* in sap, containing 1.9, 2.5, and 4.6 x 10^6 cells per ml, were prepared and irradiated while flowing as a 0.5 cm layer over a 30-watt germicidal lamp (Fig. 1). Three different flow rates, 26, 33, and 40 ml per second, were employed. After the system had been equilibrated (suspension flowing for a few minutes), samples of the eluate were taken for estimation of the number of survivors. Aliquots of the sample were mixed with acidified Sabouraud agar after dilution and plating, and survivors counted after 4 days at 80.6° F.

RESULTS AND DISCUSSION

A wide range of survival values was required which could be treated statistically. The dosages which gave this spread of data were established empirically. For the two *Pseudomonas* strains it was found that a 15-watt germicidal lamp mounted 100 cm from the petri plates gave the desired range in data when exposures of 15, 30, 45 and 60 sec were employed.

Table 1 shows the effects of the variables tested (duration of irradiation, method of exposure, holding period subsequent to irradiation, and incubation temperature) on destruction of *Pseudomonas*-25 by ultraviolet radiation. An analysis of variance of these data is given in Table 2 which shows that,

TABLE 1

The effects of method of irradiation, holding for 5 hours at 41° F, and incubation temperature on the reduction in numbers of reproducing cells of Pseudomonas-25 exposed for various lengths of time to a 15-watt germicidal lamp

Method of irradiation	Hours held at 41° F	Incubation temperature ° F	Irradiation time, seconds			
			15	30	45	60
			Percent reduction			
Continuous	0	41	9.3	66.8	88.6	97.8
	0	80.6	28.3	60.9	82.1	92.3
	5	41	26.8	72.8	89.1	97.1
	5	80.6	32.0	63.9	82.4	91.3
Discontinuous	5[1]	41	13.8	39.6	69.9	51.7
	5[1]	80.6	19.7	35.1	64.1	87.2

[1] Two—2½-hour periods.

TABLE 2

Results of the analysis of variance of data obtained with ultraviolet-irradiated Pseudomonas-25 cells (table 1)

Source	Degrees of freedom	Sum of squares	Mean square	F
Dosage (D)	3	15,255.39	5,085.13	66.84[1]
Method (M)	2	2,177.67	1,088.84	14.31[1]
Temperature (T)	1	10.20	10.20	0.13
D x M	6	463.78	77.30	1.02
D x T	3	360.66	120.22	1.58
M x T	2	143.66	71.83	0.94
D x M x T (error)	6	456.50	76.08	.....
Total	23	18,867.86		

[1] Significant at 1% level.

of the main effects, dosage and method were statistically significant at the 1% level, having F values of 66 and 14, respectively. Since the analysis of variance shows that no interactions exist between main effects, the effects of dosage and method are independent and clear-cut. As anticipated, increases in exposure times (dosage) had the most pronounced effect on the reduction of numbers of reproducing cells, regardless of the irradiation method employed. Table 1 also shows that a more effective reduction in cells was accomplished by the continuous method of exposure to ultraviolet than by the discontinuous method. Further, it can be seen that no significant influence resulted from holding survivors at 41° F for 5 hr folowing radiation.

Since temperature of incubation had no significant effect on the reduction of reproducing cells of *Pseudomonas-25*, it can be concluded that survivors recovered equally well at either low (41° F) or moderate (80.6° F) incubation temperatures.

Table 3 shows the data obtained when cells of *Pseudomonas*-11 were irradiated and the analysis of variance of these data is given in Table 4. As observed with *Pseudomonas-25*, only dosage and method of irradiation (main effects) had statistical significance at the 1% level on the reduction in numbers of reproducing cells (Table 4). Some statistical interaction is noted between dosage and method. As was the case for the *Pseudomonas-25*,

TABLE 3

The effects of method of irradiation, holding for 5 hours at 41° F, and incubation temperature on the reduction in numbers of reproducing cells of Pseudomonas-11 exposed for various lengths of time to a 15-watt germicidal lamp

Method of irradiation	Hours held at 41° F	Incubation temperature, ° F	Irradiation time, seconds			
			15	30	45	60
			Percent reduction			
Continuous	0	41	36.8	64.9	84.6	96.0
	0	80.6	37.4	69.3	84.3	94.0
	5	41	26.9	56.0	79.3	89.5
	5	80.6	28.6	60.9	80.9	89.1
Discontinuous						
	5[1]	41	19.2	39.6	55.2	79.3
	5[1]	80.6	27.1	42.6	59.1	74.5

[1] Two 2½ hour periods.

TABLE 4

Results of the analysis of variance of data obtained with ultraviolet-irradiated Pseudomonas-11 cells (table 3)

Source	Degrees of freedom	Sum of squares	Mean square	F
Dosage (D)	3	11,265.04	3,755.01	1,009.41[1]
Method (M)	2	1,892.46	946.23	254.36[1]
Temperature (T)	1	17.51	17.51	4.71
D x M	6	214.08	35.68	9.59[1]
D x T	3	38.19	12.73	3.42
M x T	2	3.50	1.75	0.47
D x M x T (error)	6	22.34	3.72	.....
Total	23	13,453.12		

[1] Significant at 1% level.

TABLE 5

Results of the analysis of variance of the comparison between data obtained for strains 11 and 25 of Pseudomonas (Tables 1 and 3)

Source	Degrees of freedom	Sum of squares	Mean square	F
Strains (S)	1	3.26	3.26	0.89
Dosages (D)	3	26,252.04	8,750.68	2,397.45[2]
Methods (M)	2	3,830.17	1,915.09	524.68[2]
Temperature (T)	1	27.76	27.76	7.61[1]
S x D	3	268.38	89.46	24.51[2]
S x M	2	239.94	119.97	32.87[2]
S x T	1	0.41	0.41	0.11
D x M	6	448.98	74.83	20.50[2]
D x T	3	151.62	50.54	13.85[2]
M x T	2	83.12	41.56	11.39[2]
S x D x M S x D x T S x M x T D x M x T S x D x M x T	(error) 23	838.55	3.65	.....
Total	47	32,144.23	.....	.....

[1] Significant at 5% level.
[2] Significant at 1% level.

continuous exposure resulted in the greatest reduction of cells, and the effect of holding at 41° F following irradiation did not appreciably increase the number of survivors. Likewise, incubation temperature had no effect on the number of survivors of irradiated cells. The data of Tables 1, 2, 3, and 4 indicate that there may be an effect due to bacterial strain difference. Since all conditions tested were identical, a statistical comparison was made of the data for the two bacterial strains and is given in Table 5. Since the F value for strains is not significant, it was concluded that strains 11 and 25 were equally susceptible to ultraviolet radiation.

Preliminary experiments showed that *C. albidus* was much more resistant to ultraviolet radiation than were the bacteria. Therefore, a stronger light source, 30-watts, mounted only 35 cm above the petri plates was used.

The results and the analysis of variance of ultraviolet-irradiated *C. albidus* cells are given in Tables 6 and 7. In contrast with the pseudomonads, the

TABLE 6

The effects of method of irradiation, holding for 5 hours at 41° F, and incubation temperature on the reduction in numbers of reproducing cells of Cryptococcus albidus exposed for various lengths of time to a 30-watt germicidal lamp

Method of irradiation	Hours held at 41° F	Incubation temperature, ° F	Irradiation time, seconds			
			15	30	45	60
			Percent reduction			
Continuous	0	41	64.5	73.9	81.5	87.2
	0	80.6	75.4	81.1	89.0	95.2
	5	41	63.2	73.2	86.0	88.9
	5	80.6	74.9	82.4	92.5	96.1
Discontinuous	5[1]	41	67.6	71.8	85.8	91.4
	5[1]	80.6	77.2	78.9	90.8	94.6

[1] Two 2½ hour periods.

TABLE 7

Results of the analysis of variance of data obtained with ultraviolet-irradiated Cryptococcus albidus cells (Table 6)

Source	Degrees of freedom	Sum of squares	Mean square	F
Dosage (D)	3	1,770.68	590.23	766.53[2]
Method (M)	2	8.14	4.07	5.28[1]
Temperature (T)	1	361.15	361.15	469.02[2]
D x M	6	31.95	5.32	6.90[1]
D x T	3	20.29	6.77	8.79[1]
M x T	2	7.11	3.56	4.62
D x M x T (error)	6	4.61	0.77	.....
Total	23	2,203.93		

[1] Significant at 5% level.
[2] Significant at 1% level.

only main effects statistically significant at the 1% level, and with no accompanying interactions, are incubation temperature and dosage. At the 5% level, methods as well as two interactions (between dosage and methods and between dosage and temperature) were statistically significant. Even so, the important main effects are dosage and temperature. Also, the 5 hr holding period at 41° F after continuous exposure had no effect on the number of survivors.

The striking feature which distinguishes these results from those observed with the bacteria was the effect of incubation temperature on survivors. With *C. albidus* a consistent and significantly lower percent reduction was observed at the lower temperature, 41° F. This is consistent with the enhanced recovery of ultraviolet-irradiated *E. coli* under conditions suboptimal for the growth of unirradiated cells reported by Alper and Gillies *(1)*.

The effectiveness of the destruction of *C. albidus* by irradiation when the liquid in which they are suspended is caused to flow at different rates over a 30-watt germicidal lamp is given in Tables 8 and 9. The effects of only two variables, flow rate and concentration of cells, were studied. Of these only flow rate (time of exposure) had statistical significance. Table 8 shows that the germicidal effect decreases as the flow rate increases. Initial cell concentration had no effect on percent reduction (Table 9).

From the above it is immediately suggested that ultraviolet radiation can be used both to disinfect sap as it is put into storage and to suppress growth during storage. The practical application of ultraviolet radiation would be to flow all sap over a germicidal lamp as it is being run into storage tanks and to have a germicidal lamp mounted in the tank to keep the sap sterile.

TABLE 8

The effect of flow rates and initial concentration on reduction in numbers of Cryptococcus albidus cells irradiated with ultraviolet in a moving liquid

Flow rate per second	Initial concentration per ml		
	1.9×10^6	2.5×10^6	4.6×10^6
	Percent reduction		
26 ml	52.6	52.4	55.6
33	47.4	50.4	49.8
40	34.8	33.7	39.3

TABLE 9

Results of the analysis of variance of data obtained with Cryptococcus albidus cells irradiated with ultraviolet in a moving liquid (Table 8)

Source	Degrees of freedom	Sum of squares	Mean square	F
Rate of flow (R)	2	504.55	252.28	97.03[1]
Concentration (C)	2	18.69	9.35	3.60
R x C (error)	4	10.38	2.60	.....
Total	8	533.62		

[1] Significant at 1% level.

or low in microbial count, during storage. It should be emphasized that if the contaminating load is high at the time the sap is collected, the quality of the sap will not be improved by ultraviolet irradiation, since the undesirable effects resulting from microbial activity would have occurred. The utility of germicidial lamps lies in its use for the disinfection, prior to storage, of slightly or moderately contaminated sap and in suppressing growth and deleterious fermentations during storage.

SUMMARY

A study was made of the effects on the reduction of living cells of two bacterial and one yeast strain suspended in maple sap when exposed to ultraviolet radiations of different intensities and for different lengths of time.

The two bacterial strains were equally sensitive to destruction by ultraviolet irradiation but were more sensitive than the yeast.

As the time of exposure to irradiation increased, there was a corresponding increase in the reduction of living cells for all organisms, regardless of the method of irradiation employed.

The two bacterial strains exposed to ultraviolet radiation by the discontinuous method were less effectively killed than when they were exposed by the continuous method, demonstrating that the total effect of mild radiation dosages is not cumulative. Destruction of the yeast strain was the same, however, by both methods of static irradiation.

Recovery from radiation damage during a 5 hour holding period at 41° F did not result in any significant increase in number of survivors for the yeast and bacteria.

The temperature of incubation, 41° F or 80.6° F, following irradiation had no effect on survivor plate counts to the two bacterial strains. The yeast strain, on the other hand, showed a higher recovery when incubated at the lower temperature.

In the flowing system, greatest reduction of the yeast occurred with the slowest flow rate (increased dosage), irrespective of initial cell concentration.

The practical significance of these results is discussed.

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ANALYSIS OF VOLATILE FOOD FLAVORS BY GAS-LIQUID CHROMATOGRAPHY. I. THE VOLATILE COMPONENTS FROM DRY BLUE CHEESE AND DRY ROMANO CHEESE

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Gas-liquid chromatography has been used by numerous investigators *(4, 7, 8, 9, 15, 16)* to study various aspects of food flavor analysis. These reports indicate that this analytical technique has considerable merit for determining the number and nature of the volatile flavor and odor substances obtained from various foods.

Objective of the current program, which concerns the chemistry of food flavors, has been to evaluate the usefulness of gas-liquid chromatography as a means of both resolving and isolating food flavor components. To this end, the volatile material obtained from dry Blue cheese and dry Romano cheese preparations was used. These substances were chosen for their simplicity in preparing the volatiles and because there is a considerable amount of information in the literature concerning the composition of the flavor and odor components of natural Blue cheese. From such information it was possible to evaluate the data obtained in our current studies.

In this respect Jackson and Hussong *(7)* have recently described the gas-liquid chromatography of various secondary alcohols obtained from Blue cheese. Earlier knowledge concerning the carbonyl compounds in such cheese has been reported by Bassett and Harper *(2)*, Morgan and Anderson *(12)* and Patton *(13)*. The various substances were resolved by paper and/or column chromatography of the 2, 4 dinitrophenyl hydrazones. These in turn were characterized by additional means.

Bassett and Harper *(2)* and Harper *(10)* have indicated that Romano cheese contains large quantities of free fatty acids as well as some acidic and neutral carbonyl compounds. The presence of the acids ketoglutaric, pyruvic, and acetoacetic were indicated in their studies.

Results of the present investigation have shown that gas-liquid chromatography is a powerful and most useful means not only for analyzing and isolating food flavors and odors but also the technique appears to offer considerable promise as a means of quality control analysis.

EXPERIMENTAL

Volatile material from dry Blue cheese[a] and dry Romano cheese[b] preparations was concentrated by a low-temperature, vacuum-distillation method similar to that described by Stahl *(16)*. The flask containing the dry cheese was immersed in a water bath held at 60° C. The volatile neutral and acidic substances, as well as moisture, were retained in the first 2 traps chilled in a Dry Ice-ethanol bath. A third trap immersed in liquid nitrogen retained the liberated ammonia and possibly low boiling amines.

[a] Blue cheese flavoring: A commercial product containing Blue cheese dried on a milk product obtained from Whitson Products, New York.
[b] Obtained from Sargento Company, Elkhart Lake, Wisconsin.
[c] Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.
[d] Johns Manville Celite Division, New York.
[e] Carbide and Carbon Chemicals Co., New York.

The aqueous solution from Blue cheese containing the neutral and acidic fractions was saturated with sodium chloride and the pH adjusted to 8.5 by the addition of 10% sodium carbonate solution. The neutral fraction was removed by ether extraction. The ether solution was dried over anhydrous calcium sulfate and the solvent removed at 35-40° C under a gentle stream of nitrogen. Loss of very volatile compounds may have occurred. The product from 10 kg of dry Blue cheese was a tan-colored liquid having a volume of 1.0 ml and a very strong odor of Blue cheese. The chromatographic pattern of this neutral fraction was determined.

The volatile acids were recovered from the alkaline aqueous solution by adjusting the pH to 1.0 with 18N sulfuric acid and then extracting with ether. The ether solution was dried and the solvent removed under nitrogen. The acidic fraction was a tan-colored liquid having a volume of 0.4 ml and an odor resembling butyric acid.

The aqueous solution of the volatile substances from Romano cheese was saturated with sodium chloride and the volatiles removed by extraction with ether and isopentane. The solvent solution was dried wtih anhydrous sodium sulfate and the solvent removed from the dry solution at about 35° C in a stream of nitrogen. About 0.3 ml of a yellow-colored liquid with a strong Romano cheese odor was obtained from 1 kg of cheese. The chromatograph pattern of these total volatiles was determined.

Total volatiles from Romano cheese were separated into the acidic and neutral components by the same method as used with the Blue cheese extract. The chromatograph pattern of the ether solution of the neutral fraction was determined. No components were evident suggesting that the Romano cheese volatiles were all acidic.

Volatile acids from both the Blue cheese and the Romano cheese preparations were methylated with diazomethane according to Feiser *(6)*. This reaction was carried out using diazomethane prepared from a Carbitol solution of Diazald.[c] The compositions of the methyl ester fractions were also studied by gas-liquid chromatography.

The gas-liquid chromatography was carried out using an instrument built in our laboratory. Its design was similar to that described by Dimick and Corse *(4)*. The column was 10 ft of ¼ inch O.D. aluminum tubing. The packing was acid washed 30-50 mesh Chromasorb[d] upon which was adsorbed UCON 50 HB-5100.[e] This substrate was present as 14% of the total column packing for Blue cheese and 28% for Romano cheese fractions. Chromatography was carried out at 150° C using a corrected helium flow of 50-60 ml/min. Ten to 20 microliter aliquots of the neutral fraction and of the methyl esters were repeatedly chromatographed to obtain sufficient quantities of the various components for further study. These components were recovered without the aid of solvent in glass traps chilled in a Dry Ice-ethanol bath. These traps were similar to those described by Dimick and Corse *(4)*.

With the Blue cheese volatiles the initial identification of the isolated components was made by infrared spectroscopy. To this end the samples were dissolved in carbon tetrachloride and the solution placed in micro cells for analysis on a Beckman IR-4 instrument. Subsequently the ketones were converted into their 2,4 dinitrophenyl hydrazones by reacting them with a 2N hydrochloric acid solution containing 2.0 gm of 2,4 dinitrophenyl hydrazine per liter. The derivatives precipitated readily. They were recovered and recrystallized from aqueous methanol to a constant mp. Similar derivatives were prepared from authentic ketones which had been subjected to gas-liquid chromatography. The physical characteristics of the chromatographed ketones were identical to those described in the literature. In this way it was reasonably certain that gas-liquid chromatography had not produced artifacts. Physical properties and paper chromatographic characteristics of the isolated and authentic substances were compared for final characterization. The paper chromatography of the 2,4 dinitrophenyl hydrazones was carried out according to the methods described by Lynn, Steele and Staple *(11)* and Ellis, Gaddis, and Currie *(5)*.

Alcohol identification was by means of chromatographic data, infrared spectroscopy, and the properties of the 3,5 dinitrobenzoate prepared according to Cheronis and Entrikin *(3)*. In the characterization of one of the alcohols, heptanol-2, an authentic specimen was prepared by the sodium reduction of heptanone-2 *(1)*. The product was purified first by distillation followed by gas-liquid chromatography.

Only retention times were determined on the methyl esters obtained from Blue cheese acids.

With Romano cheese volatiles isolated fractions were also examined by infrared spectroscopy for their initial identification. Retention values and infrared patterns of

known materials were then obtained and compared for establishing the identity of the fractions.

RESULTS

A. Blue Cheese. Six of the 29 dry Blue cheese neutral volatiles were major components accounting for 93% of the chromatographed material. A reproduction of the chromatogram of the neutral volatile components is shown in Figure 1. The numbered peaks are the major ones recovered for identification.

The isolated fractions were initially shown by infrared spectroscopy to be ketones and alcohols. The ketones were further characterized by the properties of their 2, 4 dinitrophenyl hydrazones. Tables 1 and 2 present mp data and paper chromatographic properties for these respectively.

Two of the isolated, major components from the dry Blue cheese were alcohols. One of these, component Number 10, was found to be heptanol-2. The gas-liquid chromatographic properties and the characteristics of the 3, 5 dinitrobenzoate of this was found to be identical to authentic heptanol-2. Table 3 presents the data on this alcohol.

The second alcohol was component Number 20. It was recovered in an amount barely sufficient for infrared analysis. This information coupled with the gas-liquid chromatographic data strongly suggested that this substance was nonanol-2.

The acidic components isolated from the dry Blue cheese volatiles were chromat-

TABLE 1

Melting point characteristics of DNPH derivatives of ketones isolated from dry Blue cheese

	Dinitrophenyl hydrazones melting points			
Component No.	Cheese component mp, °C	Authentic specimen mp, °C	Mixed mp, °C	Identity
7	73.0	74.0	74.0	Heptanone-2
16	56.5	56.5	56.5	Nonanone-2
17	47.5	.....	.....	Mixed ketone[1]
25	63.0	64.0	64.0	Undecanone-2

[1] Component No. 17 was a mixed ketone containing nonanone-2 and an unidentified ketone. Infrared spectroscopy suggested this molecule may contain terminal unsaturation.

TABLE 2

Paper chromatographic characteristics of DNPH derivatives of dry Blue cheese ketones

	R_f relative to C11 methyl ketone		
Component No.	Blue cheese ketone	Authentic ketone	Identity
7	0.69	0.69	Heptanone-2
16	1.67[1]	1.67	Nonanone-2
17	0.63		
25	0.98	1.00	Undecanone-2

[1] Paper chromatographic method of Ellis, Gaddis and Currie *(5)*. The other compounds were chromatographed according to Lynn, Steele and Staple *(11)*.

TABLE 3

Characteristics of dry Blue cheese volatile component number 10

Gas-liquid chromatographic retention relative to C-8 methyl ketone		Melting point data of 3, 5 dinitrobenzoate mp, °C		
Component No 10	Heptanol-2	Component No 10	Heptanol-2	Mixed mp
0.89	0.89	48	49	48

ographed as their methyl esters. None was isolated for conclusive identification. However, the relative retention values of these and the methyl esters of known acids strongly suggested the nature of the cheese acids.

Table 4 presents the data which were obtained on the major ten of the 26 peaks seen in the gas-liquid chromatogram.

TABLE 4

Gas-liquid chromatographic characteristics of the methyl esters of volatile acids derived from dry Blue cheese

Component No.	Per cent of total	Retention relative to methyl caproate	Estimated boiling point ° C	Tentative identification
1	0.83	0.37	102	Butyric
2	0.70	0.45	113	Isovaleric
3[1]	0.42	0.80	138	
4	14.40	1.00	149	Caproic
5	1.40	1.91	177	Heptanoic
6	26.00	2.73	193	Caprylic
7[1]	19.60	4.30	208	.....
8	3.40	5.26	213	.....
9	29.50	7.35	224	Capric
10	2.70	8.55	228	.....

[1] Components Numbers 3 and 7 originated from the Carbitol during the preparation of the diazomethane.

B. Romano cheese. The chromatographic characteristics of the components of the total volatiles from Romano cheese are given in Table 5 and a reproduction of the

TABLE 5

Gas-liquid chromatographic characteristics of dry Romano cheese volatiles

Component No.	Retention relative to heptaldehyde	Estimated boiling point ° C	Identity	Relative retention of known material
1	0.53	73	.....	
2	0.99	103	.....	
3	1.57	123	.....[1]	
4	3.65	163	Butyric Acid	3.65
5	10.9	202	Caproic Acid	10.95

[1] Might be acetic acid which has a relative retention time of 1.41.

chromatogram is shown in Figure 2. Only 2 major components appeared. These were identified as butyric and caproic acids. The other acids present were not sufficiently volatile at 150° C to pass through the column in the time used.

No neutral components were evident.

The methyl ester fraction of the acidic components contained 14 components. The characteristics of these components are given in Table 6; their chromatogram is reproduced in Figure 3. Relative retention times are given in relation to heptaldehyde which has the same retention time as methyl caproate.

The major components 5, 9, 11, 13 and 14 were isolated and examined by infrared analysis. These infrared spectra together with the retention times of these in comparison to known compounds definitely indicated components 5, 9, 11 and 14 to be the methyl esters of butyric, caproic, caprylic and capric acids. Component 13 is derived from Carbitol which was used in the preparation of diazomethane. This distilled over with the diazomethane during preparation. The other components were too small in quantity for identification. Many of these as well as the 4 identified esters were also evident in the methyl ester fraction of the Blue cheese volatiles. Component 13 (Carbitol) was also present in the Blue cheese ester preparation. Component 8 may have come from the methylation mixture also as it appeared to be present in the Blue cheese preparation.

TABLE 6

Gas-liquid chromatographic characteristics of the methyl esters of the volatile acids from dry Romano cheese

Component No.	Per cent of total	Retention relative to heptaldehyde	Estimated boiling point °C	Identity	Relative retention of known material
1	1.44	0.14	56	.....[1]	
2	0.04	0.18	70	.....	
3	0.01	0.22	77	.....	
4	0.01	0.28	90		
5	20.30	0.35	100	Methyl butyrate	0.351
6	0.07	0.49	111	.....	
7	0.21	0.62	125	.....	
8	0.14	0.81	137	.....	
9	58.20	1.02	147	Methyl caproate	1.00
10	0.41	1.97	177	.....	
11	5.84	2.72	192	Methyl caprylate	2.76
12	0.82	3.92	209	.....	
13	9.36	4.56	215	Carbitol[2]	
14	3.13	7.43	225	Methyl caprate	7.33

[1] Could be methyl acetate which has a relative retention time of 0.141.
[2] Present in the methylation solution.

DISCUSSION

In this study the components separated and identified from Blue cheese are in part the same as those described by Patton *(13)* and Morgan and Anderson *(12)*, and Bassett and Harper*(2)*. These authors isolated various Blue cheese carbonyls as the 2, 4 dinitrophenyl hydrazones which were then separated and identified by paper and/or column chromatography. Recently, Jackson and Hussong *(7)* described the identification by gas-liquid chromatography of several secondary alcohols derived from Blue cheese.

These investigators have shown the presence in natural Blue cheese of the same ketones and alcohols as have been found in and isolated from the volatile material obtained from the dry Blue cheese. The studies on the natural cheese showed the presence of acetone and pentanone-2. Acetone did not appear to be present in the volatiles from the dry cheese but pentanone-2 may be one of the early minor components shown in Figure 1.

No neutral components were evident in Romano cheese volatiles. Their absence may have been caused by the drying of the cheese sample in its preparation.

The acidic carbonyl compounds found present in Romano cheese by Bassett and Harper *(2)* did not appear to be present as major components, although some of the minor methyl esters may have been derived from these acids.

In the present work it is easily observed that the dry Blue cheese volatile material is quite complex and that gas-liquid chromatography offers a valuable method for the resolution and ultimate isolation of many of the individual components.

One additional point should be mentioned in regard to gas-liquid chromatography of food flavors and odors. This pertains to quality control analysis as was suggested by Sloman and Borker *(15)*. In this respect a chromatogram of a standard flavoring material such as cheese could be considered a flavor

"profile" against which other preparations could be checked and compared for the number and concentration of components.

SUMMARY

Gas-liquid chromatography has been used to study the composition of the neutral and acidic, volatile material derived from dry Blue cheese and Romano cheese preparations.

The major components of the 29 neutral substances of Blue cheese were isolated and identified by auxiliary means. These components were heptanone-2, nonanone-2, undecanone-2, an unidentified mixed ketone, heptanol-2 and nonanol-2.

The Blue cheese acids were chromatographed as their methyl esters. Of the 26 chromatogram peaks ten were predominant in quantity. These were tentatively identified in part through comparative gas-liquid chromatographic data of known methyl esters. The major acids appear to possess four, five, six, seven, eight and ten carbon atoms.

The major components present in the volatiles obtained from Romano cheese were straight chain acids of four, six, eight and ten carbon atoms.

This method of analysis appears to be a most useful means of analyzing complex flavor substances and for the isolation of resolved components. It is believed that this analytical technique offers considerable promise as a means of determining flavor "profile" and as an added means of product control analysis.

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THE CAROTENOIDS OF JAPANESE PERSIMMONS

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The common commercial, or Japanese, persimmon, *Diospyros kaki*, is a rich source of carotenoids. In connection with this laboratory's interest in alterations of carotenoids of processed fruits, good sources of some of the more unusual carotenoid pigments were needed. It was considered possible that the persimmon is a good source of some of these pigments. This paper reports data on the composition of the carotenoid pigment mixture of this fruit.

The cartenoids of the Japanese persimmon, or kaki, were examined by Karrer *et al (8)* in 1932; an ester of zeaxanthin, lycopene, and "carotin" was found. Schön in 1935 *(9)* reported the presence of cryptoxanthin, lycopene, β-carotene, α-carotene, zeaxanthin, and violaxanthin in dried fruits of *Diospyros costata*, which is sometimes classified as a variety of *Diospyros kaki (1)*. The carotenoids of the Japanese persimmon have now been fractionated by means of countercurrent distribution into hydrocarbons, monols, diols, monoepoxide diols, diepoxide diols, and polyols *(2, 4)*. (Epoxide here refers to both 5, 6- and 5, 8-epoxides; they were referred to in previous publications by the present author as epoxides and furanoxides, respectively.) These groups were then separated by chromatography into a total of 26 constituents, not including cis-isomers. In addition to the substances reported by earlier workers, antheraxanthin, lutein, zeta-carotene, and neoxanthin were found to amount to 2% or more of the total carotenoids. Persimmons were found to be an excellent source of cryptoxanthin and to a lesser extent of zeaxanthin and antheraxanthin.

EXPERIMENTAL

The fruit used in this investigation was of the Hachiya variety, and was obtained in December, 1956, from the Plant Introduction Gardens of the U. S. Department of Agriculture, Chico, California. The fruits employed for the carotenoid extraction were firm or slightly soft, with no green color. The entire edible portion was used, including the skin but not the seeds.

Portions of 250 g of the sliced fruit were blended for several minutes with 250 ml of water containing 1 g of basic magnesium carbonate. The blend was mixed with 500 ml of methanol and 35 g of Celite per batch. After standing about 30 min. the mixture was filtered on a Buchner funnel precoated with Celite. The filter cakes were blended briefly with acetone, filtered on a sintered glass funnel and washed with acetone until the washings were colorless. The acetone extract was worked up as previously described *(2, 7)*. One of the two batches of the carotenoid extract was saponified *(2)*.

The total carotenoid content of the unsaponified extract was found to be equivalent to 54 mg per kg of fruit (as β-carotene) when measured in an Evelyn colorimeter with a 440 mmc filter. The carotenoid content of the saponified extract (the unsaponifiable fraction in the saponification of fats) was about 1% lower; this loss was due, at least in part, to the presence of acidic pigments.

Countercurrent distribution runs were carried out as previously described *(2)* in an all-glass Craig apparatus. The system petroleum ether-99% methanol, with 100 transfers, was used with aliquots of both the unsaponified and the saponified preparations, representing 150 and 500 g of fruit, respectively. A portion of the saponified extract repre-

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senting 500 g of fruit was used in a 200-transfer run with the system benzene-petroleum ether-87% methanol (1:1:1.15 by volume). By the use of these two solvent systems, the carotenoids were separated into hydrocarbons, monols, diols, monoepoxide diols, diepoxide diols, and polyols *(2)*. These fractions were then chromatographed on columns of magnesia (Westvaco No. 2642) plus Celite, using eluents as previously described *(3)*. Spectral absorption curves were run in a Cary recording spectrophotometer, Model No. 14.

RESULTS AND DISCUSSION

Results of the countercurrent distribution runs with the system petroleum ether—99% methanol are summarized in Table 1. About 84% of the total

TABLE 1

Countercurrent distribution of carotenoids from Japanese persimmons with the system petroleum ether—99% methanol

Number of hydroxyl groups	Approximate % of total carotenoids		N_{100}[1]	
	Unsaponified	Saponified	Unsaponified	Saponified
0	94	16	94	90
1	5	40	59, 65	57
2 or more	1	44	10	10

[1] Tube number of maximum per 100 transfers.

carotenoids were found to be xanthophyls, nearly half of which were monols. About 93% of the xanthophylls occurred before saponification in a fully esterified form. The distribution of the saponified carotenoids with the system benzene-petroleum ether—87% methanol as given in Figure 1 showed the presence of 21% diols, 12% monoepoxide diols, 4% diepoxide diols, and 3% polyols. Chromatography of the several fractions yielded a total of 31 bands (Table 2), 5 of which were apparently cis-isomers and may have been artifacts. The bands are listed in the order in which they were eluted from the columns, the first one being eluted first.

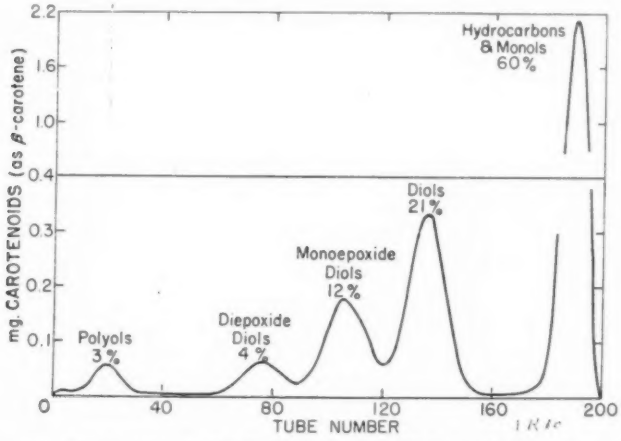

Figure 1. Countercurrent distribution of saponified carotenoids from Japanese Persimmons with the system benzene-petroleum ether-87% methanol (1 to 1 to 1.15 by volume)

TABLE 2

Carotenoids obtained from Japanese persimmons

Constituent[1]	Spectral absorption maxima, mmc[2]	Approximate %[3]	
Hydrocarbons			
Phytoene	(298), 286, 275	0.9	
Phytofluene	367, 348, 332	0.4	
α-Carotene	473, 444, 420	1.0	
β-Carotene	477, 450, (425)	6.8	
zeta-Carotene	424, 399, 378	2.7	
Mutatochrome[4]	449, 424, 402	0.26	
γ-Carotene[4]	489, 457, 431	0.8	
Lycopene	504, 472, 445	7.7	
Monols			
Hydroxy-α-carotene[4]	474, 444, 421	1.1	
Cryptoxanthin	477, 451, (427)	38.	
Cryptoflavin[4]	450, 425, 405	0.6	
Diols			
Lutein	486, 457, 433	3.9	
Zeaxanthin	492, 463, (438)	18.	
Monoepoxide diols			
Lutein 5, 6-epoxide	481, 449, 424	0.21	
Antheraxanthin	486, 457, (433)	5.8	10.0
cis-Antheraxanthin	483, 454, (430)	4.2	
Mutatoxanthin a	464, 436, (414)	0.9	1.5
Mutatoxanthin b	464, 436, 411	0.6	
Diepoxide diols			
Violaxanthin	482, 451, 426	1.1	2.7
cis-Violaxanthin	478, 447, 423	1.7	
Luteoxanthin a	459, 426, 402	0.16	0.8
Luteoxanthin b	459, 429, 405	0.4	
cis-Luteoxanthins	456, 430, 405	0.28	
Auroxanthins	434, 407, 385	0.07	
Polyols			
Valenciaxanthin[4]	402, 379, 352	0.15	
Sinensiaxanthin[4]	430, 405, 385	0.15	
Neoxanthin	479, 449, 425	2.0	
Neochrome a	459, 433, 407	0.11	
cis-Neochrome a	454, 431, 409	0.13	0.37
Neochrome b	458, 431, 406	0.06	
cis-Neochrome b	454, 431, 408	0.07	

Values in parentheses are for shoulders or humps on spectral absorption curves.
[1] Constituents in each group are listed in order of elution with the first at the top.
[2] Solvent was petroleum ether for hydrocarbons and monols, benzene for others.
[3] Based on total absorbance of each constituent at the wave length of the principal maximum.
[4] Tentative identification.

Hydrocarbons. The major constituents of this fraction were β-carotene and lycopene, both of which had been found by earlier workers *(8, 9)*, as was also a-carotene. Three bands were identified as phytoene, phytofluene, and zeta-carotene by their spectral absorption curves and position on the column on elution. Two minor bands had spectral absorption maxima close to those of mutatochrome (β-carotene-5, 8-epoxide) and γ-carotene.

Monols. The major constituent of this fraction and the largest single component was found to be cryptoxanthin, previously reported by Schön

(9). Much smaller amounts of hydroxy-α-carotene and cryptoflavin-like bands were also found.

Diols. Zeaxanthin and lutein only were found in this fraction. Zeaxanthin was reported previously *(8, 9)*, but lutein, which occurred in somewhat smaller quantity, was not. Zeaxanthin was the second most abundant carotenoid, and was present in about half the quantity of cryptoxanthin.

Monoepoxide diols. None of the constituents of this fraction was reported previously. Antheraxanthin (zeaxanthin 5, 6-epoxide) was one of the major carotenoids and was accompanied by much smaller amounts of the mutatoxanthins (zeaxanthin 5, 8-epoxides). A minor fraction on the column below antheraxanthin had spectral absorption maxima corresponding to lutein 5, 6-epoxide. The ratio of 5, 6- to 5, 8-epoxides, was considerably greater than previously found in other more acid fruits such as oranges, tangerines, and peaches *(3, 4, 5)*.

Diepoxide diols. The major part of this fraction was violaxanthin (zeaxanthin 5, 6, 5′, 6′-epoxide), which had been previously reported *(9)*. It was accompanied by considerably smaller amounts of luteoxanthins (zeaxanthin 5, 6, 5′, 8′-diepoxides) *(4)* and a very small amount of auroxanthins (zeaxanthin 5, 8, 5′, 8′-diepoxides). This change was found to have occurred to a considerable extent in soft ripe fruit such as Elberta peaches *(3)* and apricots.

Polyols. The main constituent of this fraction was neoxanthin, one of the major pigments of leaves *(10)*. Neoxanthin was found to be a monocis 5, 6-epoxide with the same conjugated double bond system as violaxanthin *(6)*. In the present work it was accompanied by much smaller amounts of the two 5, 8-epoxide isomers, for which the name "neochrome" is proposed. Strain *et al (11)* obtained two substances on heating neoxanthin in *n*-propanol in a boiling-water bath which were called neo-neoxanthins A and B. These substances appear to be the same as the substances above, called neochromes. The latter name is proposed because these substances are 5, 8-epoxide isomers of neoxanthin and not cis-isomers.

A countercurrent distribution run was carried out with the neoxanthin fraction from persimmons, using the system petroleum ether-acetone-methanol-water *(3)* (1:25:1:0.1:0.65 by volume). The N_{100} value (Table 1) was found to be 60, in good agreement with earlier values for neoxanthin from leaves (average 61). This distinguishes neoxanthin from 3 other polyols having similar spectral absorption maxima which have been obtained from orange juice or peaches, and which had N_{100} values in the above solvent system of 53, 30, and 4.

Traces were obtained of two very minor fractions which resembled valenciaxanthin and sinensiaxanthin, which were originally obtained from orange juice *(4)*.

SUMMARY

The carotenoids have been examined in Japanese persimmons *(Diospyros kaki)* of the Hachiya variety. About 84% of the total carotenoids were found to be xanthophylls, approximately half of which were monols. About 93% of the xanthophylls occurred before saponification in a fully esterified form. Excluding cis-isomers a total of 26 constituents was found, of which 17 amounted to 1% or less of the entire carotenoid mixture. No unusual

carotenoids were found in significant amount, but the fruit was found to be an unusually good source of cryptoxanthin, and a good source of zeaxanthin and antheraxanthin. These xanthophylls amounted to 38, 18, and 10%, respectively, of the total carotenoids. Other constituents, in order of decreasing abundance, were lycopene 8%, β-carotene 7%, lutein 4%, zeta-carotene and violaxanthin 3% each, and neoxanthin 2%.

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PAPER CHROMATOGRAPHIC IDENTIFICATION AND ESTIMATION OF THE FREE AMINO ACIDS IN THIRTY-TWO FRUITS

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Increasing attention is being given the role of amino acids in nutrition. This may be ascribed, at least in part, to the fact that the supplementation of foods and feeds with amino acids is becoming ever more widely practiced. The part played by amino acids in fermentation *(7)*, browning of fruits *(19)*, and in human and animal metabolism has been extensively investigated. In the field of chemotherapy, amino acids or closely related compounds have also been studied as possible inhibitors of cancer, *(21)*. These are but a few of the reasons for contemporary interest in the amino acids.

The presence of free amino acids in natural products such as fruits is admittedly well known, but reports on the number and kinds of amino acids present in each of a large series of fruits of commercial interest have not hitherto been readily available. Work on the identification of free amino acids has been limited, it appears, to a comparatively few fruits *(1, 2, 5, 6, 9, 11, 12, 13, 20)*. The present survey records for easy comparison the free amino acids in thirty-two fruits and furnishes the approximate quantitative amounts recorded. In many cases, new spots on chromatograms have been noted, indicating the possibility of the presence of amino acids not yet identified.

EXPERIMENTAL

Materials. The fruits used in this study were: apricot, cherry, japonica, nectarine, peach, plum, *(Prunus)*; avocado *(Persea)*; blue grape, red grape, white grape, *(Vitis)*; date *(Phoenix)*; fig *(Ficus)*; kumquat *(Fortunella)*; lemon, lime, Temple orange, white grapefruit, red grapefruit, orange, *(Citrus)*; mango *(Magnifera)*; papaw, southern and northern Indiana *(Asiminal)*; rhubard *(Rheum)*; pineapple *(Amanas)*; strawberry *(Fragaria)*; watermelon *(Citrullus)*; gooseberry *(Ribes)*; red raspberry *(Rubus)*; apple, green apple *(Pyrus)*; blueberry *(Vaccinium)*; persian melon *(Cucumis)*. They were purchased at local markets or were obtained from a locally produced source. Fruits that were ripe, but not past the peak of ripeness, were selected.

Procedures. In the processing of the fruit the skin or peel was removed whenever the fruit was the type in which the peel is normally removed for consumption. In all instances the seeds were removed.

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The fruit was then rinsed thoroughly in distilled water and sectioned. Approximately 1100 g of sectioned pieces (about the size of an orange section), were placed in a Waring-type blender with 70% ethyl alcohol. The extract obtained was refrigerated overnight at a temperature of 10° C, was then filtered through glass wool, and finally several times through filter paper under vacuum.

The filtered extract was poured into a column of Dowex 50-X4, (50-100 mesh) resin in the hydrogen form. Distilled water was used to dilute the salts and the amino acids were displaced with a 1N solution of ammonium hydroxide. The ammonium hydroxide was evaporated with a Rinco rotary evaporator. The final volume of ammonia free extract was approximately 350 ml.

The extract was lyophilized and a concentrated standard solution was made, a sample of which was applied to a chromatogram.

Paper chromatography was used to study the isolation and identification of the free amino acids. The technique used was after Irreverre and Martin *(10, 17)*.

Two-dimensional descending chromatograms of the concentrated extracts of the fruits were prepared using S&S 598 paper *(10, 17)*. Twenty-eight lambdas of the extract

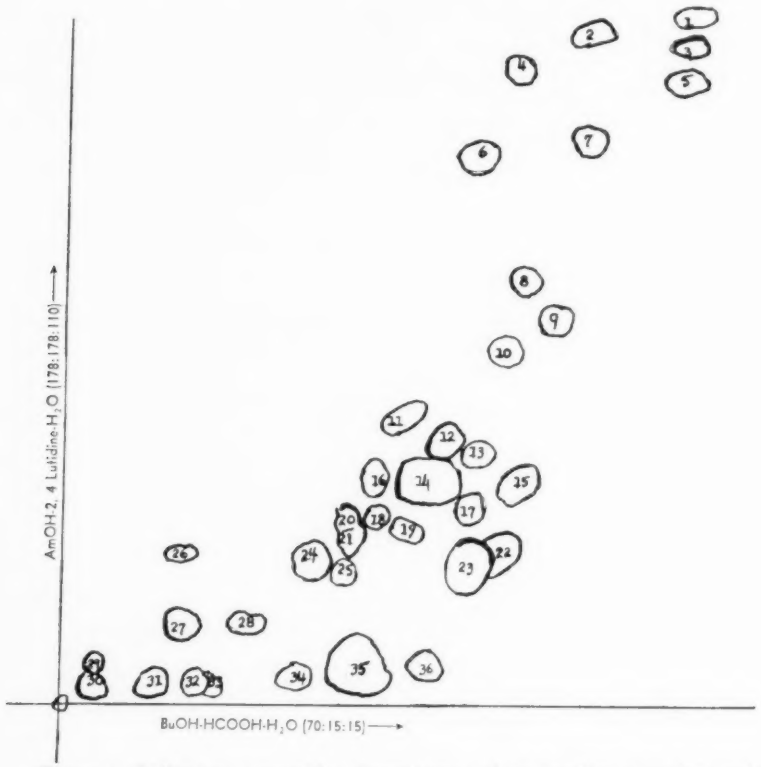

Figure 1. Indicating a composite chromatogram of the location of various amino acids.

1. norleucine. 2. phenylalanine. 3. leucine. 4. tyrosine. 5. iso-leucine. 6. tryptophan. 7. methionine. 8. valine. 9. taurine. 10. pipecolic acid. 11. ethanolamine. 12. proline. 13. alpha amino butyric. 14. alanine. 15. gamma guanidino butyric. 16. threonine. 17. beta amino iso-butyric. 18. sarcosine. 19. beta alanine. 20. serine. 21. glycine. 22. delta amino-n-valeric. 23. gamma amino butyric. 24. glutamine. 25. citrulline. 26. histidine. 27. asparagine. 28. arginine. 29. djenkolic. 30. cystine (cysteine). 31. ornithine. 32. cadaverine. 33. lysine. 34. aspartic acid. 35. glutamic acid. 36. alpha amino adipic.

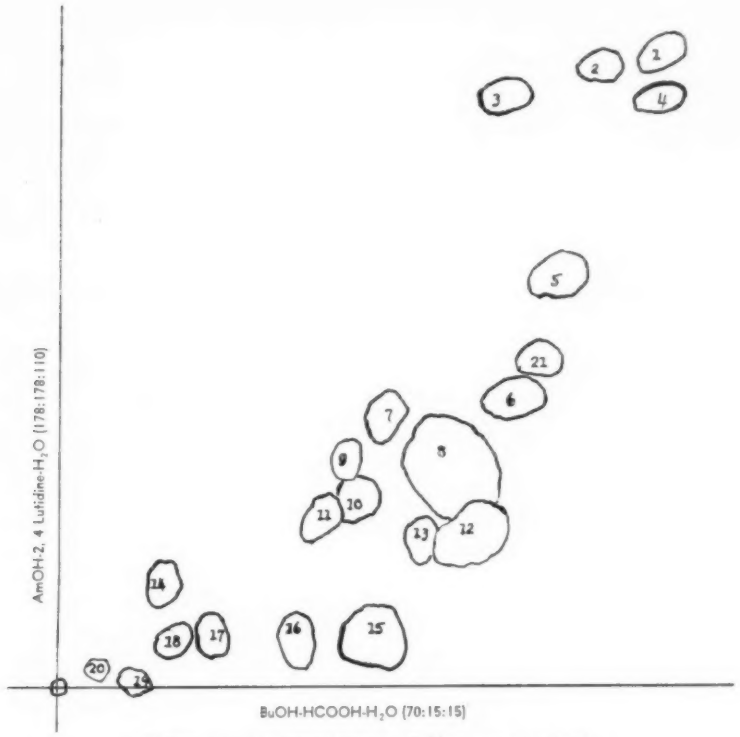

Figure 2. A chromatogram of blue grape extract.

1. leucine. 2. phenylalanine. 3. tyrosine. 4. iso-leucine. 5. valine. 6. alpha amino butyric. 7. ethanolamine. 8. alanine. 9. threonine. 10. hydroxyproline (?). 11. glycine. 12. gamma amino butyric. 13. beta alanine. 14. unknown. 15. glutamic acid. 16. aspartic acid. 17. arginine. 18. asparagine. 19. ornithine. 20. cystine (cysteine). 21. pipecolic acid.

were applied to the chromatogram and 35 ml of tert-butyl alcohol-formic acid and water (70:15:15 by volume) were employed in the first dimension. This was allowed to run until the solvent front reached the bottom of the paper (approximately 24 hrs). In the second dimension, 100 ml of tert-amyl alcohol-2, 4-lutidine-water (178:178:110 by volume) were employed. This was allowed to run for 20 hrs.

Identification and measurement. The chromatograms were dried, steamed, and then developed with .5% ninhydrin in acetone. The spots of amino acids were identified by color with ninhydrin, by standard runs of the pure amino acids, and by paper electrophoresis. Quantitative estimation was made by comparing the depth of colors and the sizes of the spots in relation to one another. The spots having the greatest amounts were labeled 3; those with less, 2; and the small light spots, 1 *(20)*. A composite chromatogram showing the location of various amino acids is presented in Figure 1.

RESULTS AND DISCUSSION

The results of the survey are summarized in Table 1. It was found that the following fruits contained the greatest number of free amino acids: avocado, fig, lemon, pineapple, blue grape, (see Figure 2), red grape, and the white grape. The cherry, lime, red raspberry, papaw (northern), and the

TABLE 1

Amino acids of thirty-two fruits

	Alanine	Alpha NH2 Butyric	Alpha NH2 Adipic	Arginine	Asparagine	Aspartic Acid	Beta Alanine	Cysteine Cystine	Gamma NH2 Butyric	Glutamic Acid	Glutamine	Glycine	Histidine	Hydroxyproline	Isoleucine	Lysine	Methionine	Phenylalanine	Pipecolic Acid	5-OH Pipecolic Acid	Ornithine	Proline	Serine	Threonine	Tyrosine	Valine	Djenkolic	Leucine	Ethanolamine	Taurine	Citrulline	Beta NH2 iso-Butyric
Apple	1				2	2		1	1	2																					
Apricot	2				2	1			1	1		1			2	1						1	1	1		2		1			
Avocado	2	1		1	1	2	1	1	2	2		1		1	1	1		1	1		1			1		2		1	1			
Blueberry	1			2						2						1												1				
Blue Grape	2	1	2		1		1		2	3		2	1		2	1	1	1	2	1		2			2	2	1	2	1				
Cherry	3				1		1	1	2	1		1			2			1	3		1	2	1		2	2	1	2		2			
Date	1				1	1	1	1	1			1				1						1							2			
Fig	2			1	1	1	1	1	3	1		1			2	2		1		2		2	1	1	1	2		2		2		
Gooseberry	3				2			1	3	3																						
Green Apple	2				1	1	1		1	2		1			1	1		1				1		1	1	1		1				
Japonica	2				1	2			2	2		1				1							1	1		2						
Kumquat	2			2	3			1	2	2		3			1			1			1	2		2	1		1	1				
Lemon	2	1		1	2	1	1	1	2	2		2	1		1	1	1	1			1	2	1		1	1	2		1	1			
Lime	2			2	2	2	1	1	3	1	1	1			2	1		2			1				2	1		2	2			
Mango	2					2	1	1	2	1	1	1			1	1		2			1				1	2	1	1	1				
Nectarine	2			2	1			1	1	1	1	1	1		1						1				1	1			1			
Orange	1			2	1	2	1		2	1		2			2	1						2		1		2		1				
Paw Paw (N. Ind.)	2			1		1	1	1	2	2	1	1			1	1		2			1	1			1	1		2	1			
Paw Paw (S. Ind.)	3					2	1	1	3	3	3	2			3	2		1				1		2	1	3		2	1	2		
Peach	2			1	1	2	1		2	1	1	1				1			1							2		2	1			
Persian Melon	2				2	2			2	2	2	2				2								1		1						
Pineapple	2	1		1	2			1	1	2	2	2	1		1	2	2	1				1		2	1	2	1	1	1			
Plum	2				1	2	2	1	2	1		1			1	1		1			1			1	1	1		1	1			
Red Grape	3			1	1		1	1	3	3	1		1		2	1	1	1			1	1			1	2	1	2	1			
Red Raspberry	3	1			2	1	2	1	2	2	2	2	1		3			1	1			2		2		3	1	2				
Rhubarb (Veg.)	1			1					1	1		1				2					1			1							
Ruby Red Grapefruit	2	1							3	2	1	1			2		1	1				1		1	1	2		1				
Strawberry	2			2	2			1	1	2		1				1	1				1											
Temple Orange	2		1		1				2	2		2				2						2		2		2	1	1	1			
Watermelon	1						1		2	2	1	1			1			1								1						
White Grape	3		1	1	1		1	1	3	3	1		2		2	1	1	2	1			2		2	2	3		2	1		1	2
White Grapefruit	1						1		2	3	1		2		1												1	1	1			

papaw (southern), mango, and plum, contained only slightly fewer amino acids.

In general the grapes and tropical fruits (except the citrus fruits) contained the highest number of amino acids. The lemon and pineapple also appeared in this class. The citrus fruits ranged between high and low in abundance of free amino acids under the conditions of this study. Fruits low in free amino acids, generally, were the melons and, with the exception of the red raspberry, the berry fruits. The smallest number of amino acids were found in the apple, blueberry, gooseberry, and rhubarb.

Lemon, pineapple, and white grape contained 9 of the essential amino acids. Red grape contained 8. The avocado and the fig contained 7 of the essential amino acids. The apple and the gooseberry by contrast appeared to contain none of the essential amino acids. The largest quantities of the essential amino acids were found in the white grape and the pineapple.

It is of interest that only alanine appeared in all the fruits. Gamma amino butyric and glutamic acid appeared in all but one fruit. Other amino acids occurring often were asparagine, glycine and valine. Alanine, gamma amino butyric, glutamic acid, and valine, when present in a given fruit, occurred in the largest quantities.

The amino acids occurring in the fewest instances were: alpha amino adipic, hydroxyproline, 5-hydroxypipecolic acid, serine, taurine, citrulline, beta amino butyric.

SUMMARY

A survey of the free amino acids of some 32 fruits was made by means of paper chromatography. The number per fruit ranged from five (blueberries) up to twenty-one amino acids for red grapes, white grapes, and pineapples. Amino acids and related compounds found in the greatest amounts in fruit were alanine, alpha amino butyric, arginine, asparagine, aspartic acid, beta-alanine, cystine, gamma amino butyric, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, leucine, and ethanolamine. The fruits containing the greatest number of free amino acids were avocado, lemon, pineapple, red grape, white seedless grape, blue grape, and fig. Red raspberry, cherry, lime, mango, papaw, and plum contained only slightly smaller amounts. The fruits which appear to contain the least amounts of amino acids were apple, blueberry, gooseberry, and rhubarb.

In general, based on the results of this survey, the grapes and tropical fruits (with the exception of most citrus) contained the highest number of amino acids.

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